Handbook of Experimental Pharmacology 248

Kathleen A. Grant · David M. Lovinger
Editors

The Neuropharmacology of Alcohol

Springer

Handbook of Experimental Pharmacology

Volume 248

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The Neuropharmacology of Alcohol

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Editors
Kathleen A. Grant
Department of Behavioral Neuroscience
Oregon Health & Science University
Portland, Oregon
USA

David M. Lovinger
Laboratory for Integrative Neuroscience
National Institutes of Health
Bethesda, Maryland
USA

ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-319-96522-2 ISBN 978-3-319-96523-9 (eBook)
https://doi.org/10.1007/978-3-319-96523-9

Library of Congress Control Number: 2018952524

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

This volume of the *Handbook of Experimental Pharmacology*, "The Neuropharmacology of Alcohol," was an exciting and challenging editorial effort. Our understanding of the pharmacology of the simple organic compound ethanol (referred to as alcohol in this volume) has flourished in the past 40 years. This volume focuses on the alcohol's central nervous system (CNS) effects and its behavioral pharmacology related to abuse potential. Many of alcohol's initial actions on brain targets that were identified in the late 1980s and early 1990s have stood the test of time and technological developments; however, far more depth and breadth has been added to our understanding of alcohol's pharmacology in the past two decades. With this reality in mind, it was difficult to assemble 20 of the most important topics for this volume. There are regrettable gaps in the neurotransmitter systems covered and the extent of phenotypic outcomes related to chronic alcohol exposure. Nevertheless, representative mechanisms of alcohol's neuropharmacology are presented, we hope to the satisfaction of the interested reader. We have organized the volume by general emphasis on neurotransmitter systems, neuropeptides, and ion channels as well as newer topics including neuroimmune systems, genomic mechanisms, and a current review of preclinical animal and human clinical studies of pharmacotherapy developments.

The gamma-aminobutyric acid (GABA) and glutamatergic systems were arguably the first receptor systems found to have the sensitivity and selectivity expected for receptor-mediated alcohol outcomes. These amino acid neurotransmitters continue to be the most studied in alcohol neuropharmacology. Furthermore, as the field continues to define both pre- and postsynaptic mechanisms, neuroanatomical specificity and GABA–glutamate interactions are becoming prominent explanations of alcohol's behavioral effects. Therefore, we highlight these recent developments with five chapters. A general overview of $GABA_A$-gated chloride channels as a target of alcohol is provided by Chandler, Overton, Ruedi-Bettschen, and Platt. This is complemented by a chapter by Lovinger on presynaptic release mechanisms implicated in G-protein coupled receptor actions on GABAeric synapses. Notable is the range of brain areas where alcohol's effects appear to alter synaptic efficacy and the potential cross talk with other drugs of abuse. The importance of alcohol's interaction with neurosteroid $GABA_A$ networks is reviewed by Finn and Jimenez. The chapter by Cuzon Carlson emphasizes the

striatum as a brain area where alcohol alters the excitatory and inhibitory balance of GABAergic and glutamatergic transmission. The contribution from Rossi and Richardson also emphasizes anatomical specificity, in this case recent data implicating the cerebellum as a site for alcohol's abuse liability through effects on $GABA_A$ channels. Next, the chapter by Hopf and Mangieri focuses on AMPA glutamate receptor (AMPAR)-mediated effects of alcohol, where advances in selective antagonists have helped illuminate subunit-specific aspects of ethanol sensitivity. Finally, another ionotropic receptor system that is prominent in the abuse aspects of alcohol is the nicotinic acetylcholine receptors. The chapter by Klenowski and Tapper provides an excellent overview of separate and common targets of alcohol and nicotine, particularly in the mesolimbic pathways with an emphasis on the apparent synergistic effects leading to comorbid addiction.

We selected two monoaminergic systems to highlight in this volume. The dopaminergic system has long occupied a predominant place in neuropharmacological aspects of addictive drugs, including alcohol. In the review by Siciliano, Karkhanis, Holleran, Melchior, and Jones, dopaminergic mechanisms that are determined under similar experimental conditions and translate across species are emphasized as a way of disentangling a complex literature. In addition to dopamine, the monoamine norepinephrine (NE) is also implicated in alcohol reinforcement mechanisms, particularly in relation to arousal, emotional regulation, and stress processes. The review by Vazey, den Hartog, and Moorman emphasizes recent findings and provides new directions for better understanding how the NE system is maladaptively altered by alcohol.

The interaction of alcohol and voltage-dependent ion channels is represented by one review on calcium channels and two reviews on potassium channels. As with the GABA and glutamate systems, alcohol research has a relatively long history with voltage-sensitive calcium channels, particularly with neurophysiological disturbances such as tremors and seizures. The review by N'Gouemo takes this complex subject and emphasizes the role of neuronal homeostasis and its disruption by chronic alcohol exposure. The large conductance voltage- and calcium-dependent potassium channel (BK) and channel interactions with alcohol are reviewed by Dopico, Bukiya, and Bettinger, with an emphasis on adaptations underlying tolerance to alcohol. Comparatively new to the neuropharmacology of alcohol is disruptions in intrinsic neuronal excitability by alcohol-induced adaptations in small-conductance calcium-activated (SK), voltage-dependent, and G-protein-activated inwardly rectifying potassium channels. These potassium channel mechanisms related to alcohol and impaired neuronal firing are reviewed elegantly by Cannady, Rinker, Nimitvilai, Woodward, and Mulholland.

The aspect of alcohol's pharmacology that is receiving renewed attention is alcohol-induced neuroinflammation involving immune signaling molecules. For this subject, we have included three reviews. The first review by Kim, McCullough, Poulson, Sanz-Garcia, Sheehan, Stravitsky, and Nagy provides the important perspective of how alcohol interferes with the hepatic immune system. The second review by Coleman and Crews focuses on innate immune signals as modulators of neurocircuitry involved in the addiction to alcohol and possibilities for new

treatment approaches. This is followed by a review from Roberto, Patel, and Bajo on the effects of key cytokines on molecular properties and synaptic transmission, particularly in the extended amygdala and hippocampus. From this collection of reviews, it is clear that targets and pharmacotherapies that emerge from the cancer biology field can be repurposed to address widespread organ dysfunction, including neuroinflammation, associated with heavy alcohol drinking.

The final set of reviews on neuropharmacological mechanisms of alcohol includes a review by Schreiber and Gilpin on the extended amygdala and corticotropin-releasing factor as a basic allosteric mechanism propagating excessive drinking. This is followed by a review of alcohol's interaction with dynorphin and orexin neuropeptide systems by Anderson, Moorman, and Becker. These two systems are closely interrelated and underlie homeostatic mechanisms that likely become dysfunctional under chronic alcohol exposure, leading to changes in motivational states that increase further alcohol consumption. The final mechanistic review is by Savarese and Lasek on genomic factors induced by alcohol that can change signal transduction mechanism and gene expression integral to long-term adaptations in chronic alcohol drinking. The emergence of new pharmacological agents that target transcriptional factors promises new directions in alcohol pharmacotherapy.

We conclude this volume with highly informative, comprehensive, and timely reviews on the practical side of alcohol neuropharmacology: approaches and outcomes of preclinical and human clinical studies of alcohol pharmacotherapeutics. The review by Egli provides background on the role of animal models as sensitive and efficient for rapid screening, but emphasizes that better translational approaches are needed to have potential pharmacotherapies retain efficacy in the arena of human outpatient treatment. The final review, by Litten, Falk, Ryan, Fertig, and Leggio, provides a history and current emphasis on developing efficacious and safe compounds for treating alcohol use disorder.

We sincerely believe that this volume provides a valuable and instructive view on the state of the art in our understanding of the depth and breadth of past, present, and future neuropharmacological research on alcohol. It remains a wonder that this simple 2-carbon alcohol can result in such complex neuropharmacology. There is no doubt that as we learn more about receptor systems, their circuitry, co-modulation, adaptive capacities, and underlying functions, we will learn more about the complex processes that result in alcohol use disorders. We hope you find this volume helpful in defining important directions of this exciting research.

Portland, OR, USA — Kathleen A. Grant
Bethesda, MD, USA — David M. Lovinger

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Part I

Neurotransmitter Systems

$GABA_A$ Receptor Subtype Mechanisms and the Abuse-Related Effects of Ethanol: Genetic and Pharmacological Evidence

Cassie M. Chandler, John S. Overton, Daniela Rüedi-Bettschen, and Donna M. Platt

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Abstract

Ethanol's reinforcing and subjective effects, as well as its ability to induce relapse, are powerful factors contributing to its widespread use and abuse. A

The original version of this chapter was revised. A correction to this chapter is available at https://doi.org/10.1007/164_2018_190.

C.M. Chandler
Graduate Program in Neuroscience, University of Mississippi Medical Center, Jackson, MS, USA

J.S. Overton · D. Rüedi-Bettschen
Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, MS, USA

D.M. Platt (✉)
Graduate Program in Neuroscience, University of Mississippi Medical Center, Jackson, MS, USA

Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, MS, USA
e-mail: dplatt@umc.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_80

significant mediator of these behavioral effects is the $GABA_A$ receptor system. $GABA_A$ receptors are the target for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS. Structurally, they are pentameric, transmembrane chloride ion channels comprised of subunits from at least eight different families of distinct proteins. The contribution of different $GABA_A$ subunits to ethanol's diverse abuse-related effects is not clear and remains an area of research focus. This chapter details the clinical and preclinical findings supporting roles for different α, β, γ, and δ subunit-containing $GABA_A$ receptors in ethanol's reinforcing, subjective/discriminative stimulus, and relapse-inducing effects. The reinforcing properties of ethanol have been studied the most systematically, and convergent preclinical evidence suggests a key role for the α5 subunit in those effects. Regarding ethanol's subjective/discriminative stimulus effects, clinical and genetic findings support a primary role for the α2 subunit, whereas preclinical evidence implicates the α5 subunit. At present, too few studies investigating ethanol relapse exist to make any solid conclusions regarding the role of specific $GABA_A$ subunits in this abuse-related effect.

Keywords

Alcohol deprivation effect · Drug discrimination · $GABA_A$ · Reinforcing effects · Reinstatement · Relapse · Self-administration · Subjective effects · Two-bottle choice

1 Introduction

Alcohol use disorders (AUDs) constitute a major public health crisis, with increases over the past several years in the numbers of individuals reporting past-year alcohol use (11% increase) and high-risk drinking (30% increase), as well as in those meeting DSM-IV diagnosis for AUD (49% increase; Grant et al. 2017). Despite the availability of both behavioral and pharmacological treatments, the majority of individuals remain untreated (Soyka and Mutschler 2016). This phenomenon likely reflects the fact that no therapeutic strategy is universally effective and that the positive outcomes associated with treatment tend not to be permanent. In turn, the lack of effective therapies suggests a need for a better understanding of the mechanisms underlying AUDs. Ultimately, greater knowledge should lead to improved treatment strategies for this patient population.

The use and abuse of alcohol are controlled by multiple effects of the drug, including its reinforcing and subjective effects, as well as its capacity to induce relapse. Ethanol's ability to enhance γ-aminobutyric acid (GABA) neurotransmission via $GABA_A$ receptors is known to be an important mechanism underlying these abuse-related effects in humans (e.g., Korpi 1994; Davies 2003; Saba et al. 2011). Numerous preclinical studies also provide evidence for the relevance of $GABA_A$ neurotransmission in the effects of ethanol. For example, nonselective ligands that increase the activity of $GABA_A$ receptors amplify several behavioral effects of ethanol (e.g., Lilijequist and Engel 1982; Söderpalm and Hansen 1998). Conversely, inhibition of $GABA_A$ receptor activity with nonspecific antagonists or

inverse agonists can attenuate both the behavioral and neurochemical effects of ethanol (Hyytiä and Koob 1995; June et al. 1998). Although the focus of this chapter is on ethanol's reinforcing, subjective, and relapse-inducing effects, it is important to note that other effects of ethanol also likely contribute to its use and abuse (e.g., tolerance, dependence, withdrawal) and that these effects may be due, at least in part, to alterations in $GABA_A$ receptor function.

2 $GABA_A$ Receptors

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the CNS. They are transmembrane ligand-gated chloride ion channels, and a number of pharmacologically and clinically important drugs including benzodiazepines, barbiturates, neuroactive steroids, and anesthetics produce their effects via $GABA_A$ receptors. These drugs allosterically modulate GABA-induced currents via distinct binding sites (Sieghart 2015). Although under some debate, there is evidence that alcohols interact directly with $GABA_A$ receptors. For example, alcohols with different numbers of carbon atoms in their backbones (i.e., methanol [3 carbons] to dodecanol [12 carbons]) potentiate the effects of GABA on $GABA_A$ receptors expressed in *Xenopus* oocytes. Interestingly, when the number of carbons exceeds 12, the alcohol is no longer able to potentiate GABA-induced current (Dildy-Mayfield et al. 1996). This lack of effect of longer-chained alcohols has been interpreted to indicate that there is a binding pocket on $GABA_A$ receptors of a defined size that can accommodate alcohols. Additional evidence supports this notion. Using site-directed mutagenesis, Mihic et al. (1997) identified regions in the second and third transmembrane segments of the α subunit of the $GABA_A$ receptor that are essential for modulation by alcohols. That is, specific mutation of particular amino acid residues markedly reduced or eliminated the effects of alcohols on the receptor. Following up on these results, another study showed that treatment of mutant receptors with propyl methanethiosulfonate, an agent that covalently binds mutated cysteine residues and occupies the putative ethanol binding site, completely abolishes enhancement of GABA current by alcohols (i.e., octanol; Mascia et al. 2000). Regardless of the specifics of the interaction between ethanol and these receptors, studies of the single-channel properties of $GABA_A$ receptors show that ethanol-induced potentiation of GABA-induced currents is due to an increase in the frequency and duration of channel opening as well as an increase in channel bursting and burst duration. Moreover, the amount of time that the channel remains in the closed state is reduced (Tatebayashi et al. 1998). The net outcome of these effects is increased ion flux through the open channel in the presence of GABA and ethanol and, ultimately, hyperpolarization of the cell and a reduced tendency to generate an action potential.

Structurally, $GABA_A$ receptors are pentamers comprised of subunits from at least eight different families of distinct proteins (6 α, 3 β, 3 γ, 1 δ, 1 ε, 1 θ, 1 π, and 3 ρ subunits); however, the majority of native receptors consist of 2 α, 2 β, and 1 γ2 subunit (McKernan and Whiting 1996; Rudolph and Möhler 2004; Fritschy and Panzanelli 2014). $GABA_A$ receptors primarily occur postsynaptically, although

there is evidence that certain subtypes may occur extrasynaptically (e.g., α5-containing subtype: Sur et al. 1999; Pirker et al. 2000; δ-containing subtype: Nusser et al. 1995; Melón et al. 2017). In addition to localization relative to the synapse, particular subunits exhibit distinct localization within the CNS (McKernan and Whiting 1996). For example, the α5 subunit is found almost exclusively in the hippocampus, the α4 subunit in the thalamus, and the α6 subunit in the cerebellum. With a slightly more promiscuous distribution, α2 and α3 subunits can be found in cortical areas, the limbic system and the spinal cord. The most widely distributed and abundant $GABA_A$ subunit is α1 which can be found throughout the brain (Pirker et al. 2000). Importantly, expression of these subunits is sensitive to and can be altered by both acute and chronic ethanol (e.g., Lewohl et al. 1996; Henby et al. 2006; Kumar et al. 2009; Olsen and Liang 2017). It is this pattern of distribution (both regionally and in response to ethanol) that likely contributes in part to the varied effects engendered by ethanol. Importantly, researchers have been able to capitalize genetically and pharmacologically on the existence of multiple subtypes (for reviews see Enoch 2008; Stephens et al. 2017) and, for example, use $GABA_A$ subtype-specific compounds to tease apart the contributions of different $GABA_A$ subunits to the abuse-related effects of ethanol (see Table 1 for a listing of pharmacological agents).

3 Reinforcing Effects of Ethanol

3.1 Models of Ethanol Reinforcement

The reinforcing effects of ethanol have been studied extensively in animals primarily using procedures in which alcohol exposure is controlled by the animal (Brabant et al. 2014). Although many different ethanol self-administration models exist, $GABA_A$ receptor subtype mechanisms have been studied primarily in the context of the two-bottle choice paradigm and operant self-administration procedures. The two-bottle choice procedure is the simplest self-administration model and allows for the assessment of ethanol's reinforcing properties through measures of ethanol consumption and preference. Typically, two bottles are available continuously to the animal, one containing an ethanol solution and the other water. Using this procedure, pharmacological manipulations can provide insight into ethanol's reinforcing properties by shifting preference toward/away from the ethanol-containing bottle or increasing/decreasing consumption of the ethanol solution. This procedure can be modified in a variety of ways including imposing an intermittent schedule to increase consumption, providing multiple bottles with varying concentrations of ethanol to inform about compulsive drinking, or assessing the strength of ethanol reinforcement by adding aversive flavors (Gilpin and Koob 2008; Brabant et al. 2014).

Operant self-administration procedures are more complex than two-bottle choice and can more directly assess a substance's reinforcing properties because animals are required to emit an arbitrary response (e.g., lever press, nose poke) to gain access to the substance. If the substance serves as a reinforcer, its delivery will increase the likelihood of the animal emitting the response again. Animals readily

Table 1 Pharmacological "tools" targeting specific $GABA_A$ receptor subunits

Compound	Classification	Relevant citation(s)
Zolpidem	α1-preferring agonist	Puia et al. (1991), Wafford et al. (1993b), Huang et al. (2000), Harvey et al. (2002), Sanna et al. (2002), and Street et al. (2004)
Zaleplon	α1-preferring agonist	Damgen and Luddens (1999) and Sanna et al. (2002)
Abercarnil	α1-preferring agonist	Lameh et al. (2000b)
CL 218,872 (3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-b]pyridazine)	α1-preferring partial agonist	Wafford et al. (1993b), Huang et al. (2000), and Harvey et al. (2002)
βCCt (β-carboline-3-carboxylate-*t*-butyl ester)	α1-preferring antagonist	Huang et al. (2000), Harvey et al. (2002), and Yin et al. (2010)
3-PBC (3-propoxy-β-carboline hydrochloride)	α1-preferring antagonist	Harvey et al. (2002) and Yin et al. (2010)
3-ISOPBC (3-isopropoxy-β-carboline hydrochloride)	α1-preferring antagonist	Tiruveedhula et al. (2015)
THIP (also gaboxadol; 4,5,6,7-tetrahydroisoxazolo-[5,4-c] pyridine-3-ol)	α4/δ-preferring agonist	Brown et al. (2002)
Neurosteroids	α4/6/δ-preferring agonist/inverse agonist	Lambert et al. (2003)
QH-ii-066 (1-methyl-7-acetyleno-5-phenyl-1,3-dihydro-benzo [e]-1,4-diazepin-2-one)	α5-preferring agonist	Huang et al. (1996, 2000)
Panadiplon	α5-selective partial agonist	Petke et al. (1992) and Lameh et al. (2000a)
XLi-093 (bis 8-ethynyl-4H-imidazo [1,5a]-[1,4] benzodiazepine)	α5-selective antagonist	Li et al. (2003)
Ro 15-4513 (ethyl 12-azido-8-methyl-9-oxo-2,4,8-triazatricyclo [8.4.0.0^{2,6}]tetradeca-1 (10),3,5,11,13-pentaene-5-carboxylate)	α5-preferring inverse agonist	Wafford et al. (1993a), Hadingham et al. (1995), Huang et al. (2000), Smith et al. (2001), Kelly et al. (2002), and McKay et al. (2004)
RY023 (*tert*-butyl 8-[(trimethylsilyl)ethynyl]-5,6-dihydro-5-methyl-oxo-4H-imidazo[1,5a]-[1,4] benzodiazepine-3-carboxylate)	α5-selective inverse agonist	Huang et al. (2000), June et al. (2001), and McKay et al. (2004)
RY024 (*tert*-butyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a]-[1,4] benzodiazepine-3-carboxylate)	α5-selective inverse agonist	McKay et al. (2004)

(continued)

Table 1 (continued)

Compound	Classification	Relevant citation(s)
L-655,708 (ethyl [S]-11,12,13,13a-tetrahydro-7-methoxy-9-oxo-9H-imidazo [1,5-a]pyrrolo[2,1-c][1,4] benzodiazepine-1-carboxylate)	α5-selective inverse agonist	Quirk et al. (1996) and Atack et al. (2006)
α5IA-II	α5-selective inverse agonist	Street et al. (2004)

respond for the delivery of ethanol, most commonly orally, but ethanol self-administration also has been demonstrated via other routes of administration (intracranial, Gatto et al. 1994; intravenous, Grupp 1981; intragastric, Fidler et al. 2006). Depending on the experimental question, operant procedures can be modified to assess, for example, demand/motivation (progressive-ratio schedule) or preference (availability of an alternate reinforcer; Gilpin and Koob 2008). Interestingly, despite the different natures of the procedures, ethanol intake in the two-bottle choice paradigm is positively correlated with operant ethanol self-administration across many rodent strains (Green and Grahame 2008).

3.2 $GABA_A$ Receptors and Ethanol Self-administration

3.2.1 α1-Containing $GABA_A$ Receptors

Evidence of a role for the α1 subunit in the reinforcing properties of ethanol has been mixed, both in rodent and nonhuman primate studies. α1 knockout mice tested under a two-bottle choice procedure showed reduced preference for ethanol, as well as a reduction in ethanol consumption, particularly at higher ethanol concentrations (Blednov et al. 2003; June et al. 2007). However, consumption of and preference for a saccharin solution also were decreased, suggesting a more general effect of deletion of this subunit on consummatory behavior. Similarly, compared to wild types, α1 knockout mice exhibited significantly lower rates of responding for an ethanol + sucrose solution, as well as a sucrose-only solution, under operant conditions (June et al. 2007). In α1 knockin mice in which the α1 subunit was rendered insensitive to potentiation by ethanol, no differences in consumption of or preference for ethanol were observed between knockin and wild-type mice (Werner et al. 2006). Interestingly, though, Yang et al. (2011) used siRNA techniques to suppress α1 gene expression in the ventral pallidum of high-alcohol-drinking rats and observed a selective decrease in binge ethanol drinking, but not binge sucrose drinking or water consumption. This latter finding is supported by studies using pharmacological approaches. Pharmacological targeting of α1-containing receptors in rats via systemic injection or infusion into the ventral pallidum of the α1-preferring antagonist 3-PBC selectively reduced operant responding for ethanol, while only altering responding for sucrose at the highest dose tested (Harvey et al. 2002). A second α1-preferring antagonist βCCt selectively reduced operant

responding for ethanol following bilateral infusion into the central nucleus of the amygdala or into the ventral pallidum in alcohol-preferring rats (Foster et al. 2004; June et al. 2003). These latter results in rats support a role for the α1 subunit in the reinforcing properties of ethanol.

In studies with nonhuman primates, the effects of the α1-preferring agonist zolpidem, as well as βCCt and 3-PBC, were evaluated in rhesus monkeys self-administering either ethanol or sucrose solutions. Although all of the α1-preferring compounds (agonists and antagonists) increased the latency to obtain the first ethanol delivery (indicating that behaviorally relevant doses were under evaluation), these compounds had no effect on consumption of either alcohol or sucrose (Sawyer et al. 2014). These results suggest that the α1 subunit may not be significantly involved in the reinforcing effects of ethanol (or consummatory behaviors). Paradoxically, 3-PBC, albeit at a higher dose than in rhesus monkeys, reduced responding, volume consumed, and dose of ethanol self-administered in baboons (Kaminski et al. 2012). Similar to rhesus monkeys, though, 3-PBC increased the latency to initiate drinking behavior. The common finding that 3-PBC increases the latency to begin drinking might provide initial evidence of a role for this receptor subtype in the drive to initiate drinking (perhaps drinking after abstinence/relapse-like drinking), rather than in "fundamental" reinforcing effects per se. Using a similar procedure in baboons and a related α1-preferring antagonist 3-ISOPBC, Holtyn et al. (2017) saw a decrease in operant responding for ethanol, as well as in ethanol intake. They also observed decreases in the number of drinks in the first drinking bout and a shortening of bout duration. Altogether, the preclinical evidence for the involvement of the α1 subunit in the reinforcing effects of ethanol remains equivocal and may be dependent on the species and/or procedure. Of note, several genetic association studies utilizing data from the Collaborative Study on the Genetics of Alcoholism found no association between alcoholism and the *GABRA1* gene encoding the α1 subunit (Song et al. 2003; Dick et al. 2005) suggesting that this subtype plays little role in this disease in humans.

3.2.2 α2/3-Containing GABA$_A$ Receptors

α2- and α3-containing GABA$_A$ receptors possess a high degree of molecular/structural homology in both the extracellular and the transmembrane domains (Whiting et al. 1999). As such, it has been difficult for chemists to develop compounds that selectively target one of these subunits over the other. It is only in the past several years that drugs have been developed that exhibit "functional" selectivity, rather than binding selectivity, for these receptor subtypes. Despite genetic association studies indicating a positive association of both *GABRA2* and *GABRA3* (genes encoding the α2 and α3 subunits, respectively) with an increased risk for developing alcoholism across varied populations (e.g., Parsian and Cloninger 1997; Covault et al. 2004; Edenberg et al. 2004; Enoch et al. 2009; Li et al. 2014; Melroy et al. 2014; Strac et al. 2015; but see Matthews et al. 2007; Covault et al. 2008), few researchers have used these drugs to investigate the role of these subunits in the abuse-related effects of ethanol. Rather, information about the potential contribution of the α2 subunit, at least, to the reinforcing effects of ethanol

comes from studies with transgenic mice. While α3 knockin mice exist (e.g., Smith et al. 2012), they have yet to be evaluated in procedures relevant to ethanol's abuse-related effects.

Studies with α2 knockout mice provide weak evidence of a role for this subunit in ethanol reinforcement and consummatory behaviors. In a two-bottle choice paradigm, for example, only female knockouts showed reduced preference and intake. However, females also tended to show lower preference than males for quinine solutions raising the possibility that the observed reduction in ethanol consumption merely reflected an enhanced aversion to bitter tastants (Boehm et al. 2004). In an operant self-administration procedure, male knockout mice did not differ from their wild-type counterparts in acquisition of self-administration, active lever presses, or number of reinforcers earned at each ethanol concentration (Dixon et al. 2012). Stronger evidence linking the α2 subunit to ethanol's reinforcing effects comes from studies with α2 knockin mice in which the α2 subunit is rendered insensitive to ethanol. Blednov et al. (2011) found that ethanol consumption and preference were generally reduced in male knockin mice under two-bottle choice procedures. Whereas in other models of alcohol consumption, males and/or females showed increased consumption and/or preference (Blednov et al. 2011). Based on the findings from genetic and rodent literature, it seems likely that the α2 subunit has some as yet clarified role in ethanol reinforcement – a conclusion that likely is not all that surprising given that α2-containing $GABA_A$ receptors can be found in midbrain dopamine neurons that comprise part of the brain reward circuitry (Okada et al. 2004).

3.2.3 α5-Containing $GABA_A$ Receptors

Studies focused on α5-containing $GABA_A$ receptors provide the most consistent evidence of a role for a particular subunit in the reinforcing effects of ethanol. Moreover, the findings regarding this subtype are convergent, spanning transgenic mice to outbred/selectively bred rat lines to nonhuman primates. In that regard, studies with male α5 knockout mice showed that the mutants preferred and consumed less ethanol than their wild-type counterparts in the absence of any differences in preference for other tastants, suggesting a specific role for this subunit in ethanol reinforcement (Boehm et al. 2004). In another study, the behavior of female α5 knockout mice was assessed in operant self-administration and two-bottle choice procedures (Stephens et al. 2005). While the knockouts did not differ from the wild types in terms of operant self-administration, the α5-selective inverse agonist Ro 15-4513 decreased drinking in both wild types and knockouts, although the drug was less effective at decreasing self-administration in the knockouts. Another α5-selective inverse agonist, α5IA-II, also was shown to reduce operant self-administration in male mice from a C57Bl X 129sv-derived line. These findings would suggest that inverse agonism at the α5-containing $GABA_A$ receptors is sufficient to reduce the reinforcing effects of ethanol but that α5 subunits are not necessary for ethanol reinforcement. Interestingly, in two-bottle choice studies, the female knockouts tended to consume less ethanol, especially at higher concentrations, endorsing a role for the α5 subunit in ethanol consumption.

Much of the transgenic mice work was inspired by earlier pharmacological studies in rats demonstrating a role for the α5 subunit in ethanol self-administration. For example, intrahippocampal infusions of the α5-selective inverse agonist RY023 reduced operant responding for ethanol but not concurrently available saccharin in alcohol-preferring P rats (June et al. 2001). Infusions of RY023 into the nucleus accumbens or ventral tegmental area had no effect on self-administration implying a specific role of hippocampal α5-containing receptors in the extended ethanol reward circuitry. Similar findings of selective attenuation of ethanol, but not water, self-administration were obtained with another selective α5-inverse agonist RY024 and in an outbred rat strain (McKay et al. 2004).

The positive findings in rodents are bolstered by a study in rhesus monkeys investigating the modulation of operant ethanol self-administration by ligands selective for α5-containing receptors (Rüedi-Bettschen et al. 2013). These authors found that L-655,708, an α5-selective inverse agonist, dose-dependently and selectively inhibited ethanol but not sucrose self-administration. Conversely, the α5-preferring agonist QH-ii-066 enhanced ethanol but not sucrose self-administration. Importantly, both the inhibition and enhancement of ethanol self-administration could be blocked by the α5-selective antagonist XLi-093 confirming a role for this receptor subtype. Together, the preclinical evidence suggests a prominent and specific role for α5-containing receptors in the reinforcing effects of ethanol. A conclusion that is supported by a significant association between *GABRA5* (the gene encoding the α5 subunit) and alcohol dependence in humans (Song et al. 2003).

3.2.4 α4/6δ-Containing GABA$_A$ Receptors

α4- and α6-containing GABA$_A$ receptors can most often be found co-expressed with the δ subunit and mediate slow, tonic neuronal inhibition. Although controversial (cf. Korpi et al. 2007), the expression of these 2 α subunits, concomitant with β3 and δ subunits, has been shown to render them sensitive to low-to-moderate ethanol concentrations and implicates them in the acute effects of ethanol often obtained via social drinking (Wallner et al. 2006, but see Korpi et al. 2007). Behavioral studies with α4 and α6 knockout mice link these subunits to some of the behavioral effects of ethanol (e.g., α4: Chandra et al. 2008; Iyer et al. 2011; α6: Homanics et al. 1997b, 1998). Unfortunately, neither mouse line has been evaluated in procedures designed to assess reinforcing effects.

α4- and α6-containing receptors have been classified as benzodiazepine-insensitive receptors due to the fact that classic benzodiazepines do not bind at these receptors. This lack of binding is driven by the absence of the γ subunit (δ subunit replaces the γ subunit) that comprises part of the benzodiazepine binding site on the receptor. Few selective ligands are available to pharmacologically probe these receptor subtypes. One exception is the ligand THIP (also gaboxadol) which was recently determined to be a partial agonist that acts preferentially at extrasynaptic GABA$_A$ receptors that co-express either the α4 or α6 subunit (Chandra et al. 2006; Herd et al. 2009). Older studies in which THIP was used to demonstrate the role of general GABA modulation in ethanol reinforcement can now be reinterpreted in light of the newly determined

selectivity profile of the compound. In that regard, THIP has been shown to enhance acquisition of voluntary ethanol consumption (Smith et al. 1992) and increase ethanol intake and preference in rats under a two-bottle choice procedure (Boyle et al. 1993), implicating the α4 and/or α6 subunits as regulators of the reinforcing properties of ethanol. However, other studies in mice (Moore et al. 2007; Ramaker et al. 2011; but see Fritz and Boehm 2014) found that THIP reduced ethanol intake in both two-bottle choice and drinking-in-the-dark procedures. Of note, though, in the latter studies THIP also appeared to reduce water intake indicating that the reductions may be due to nonselective effects of the compound. It also is not clear whether the variable effects are due to the different species under study. In a more direct evaluation of the role of α4 subunits in ethanol reinforcement, Rewal et al. (2012) used viral-mediated RNAi to decrease the expression of the α4 subunit in the nucleus accumbens core and shell in Long-Evans rats. Subsequently, operant responding for ethanol was observed to be reduced when the α4 subunit was inhibited in the shell, but not the core. Together, these findings provide initial evidence of a role for α4- and/or α6-containing $GABA_A$ receptors in the reinforcing properties of ethanol. Future studies probing the roles of these subunits could focus on the genetic models, as well as on newly developed pharmacological tools (cf. Forkuo et al. 2016).

As noted above, receptors containing the δ subunit always also contain α4 or α6 subunits. As such, the evidence for these α subunits playing a role in ethanol's reinforcing effects also applies to the δ subunit. In a more straightforward evaluation of a role for δ subunits, however, δ knockout mice have been studied in a two-bottle choice paradigm (Mihalek et al. 2001). These mice show decreased preference and consumption of ethanol. Another study used viral-mediated RNAi to decrease δ subunit mRNA and protein in the medial nucleus accumbens shell and, subsequently, observed a decrease in ethanol but not sucrose intake (Nie et al. 2011). Together, these results imply a potentially key role for the δ subunit, either alone or co-expressed in receptors with α4 or α6 subunits, in the reinforcing effects of ethanol. Interestingly, a significant association has been observed between polymorphisms in *GABRA6* (the gene encoding the α6 subunit) and alcohol use disorder across populations (Radel et al. 2005; Li et al. 2014).

3.2.5 Other $GABA_A$ Receptor Subunits

There is some evidence from genetic association studies that particular β subunits may be related to AUD-relevant phenotypes. For example, the incidence of or risk for alcoholism has been linked to varying degrees to *GABRB1*, the gene that encodes the β1 subunit (Parsian and Zhang 1999; Song et al. 2003; but see McCabe et al. 2017). Likewise, in Caucasian populations, there is evidence for a relationship between alcoholism and *GABRB3*, the gene encoding the β3 subunit (Song et al. 2003). In preclinical studies, both β1 and β2 mutant mice have been evaluated in self-administration procedures. Mice from an ethanol-averse strain with an ENU^{25}-induced L285R point mutation or a P228H spontaneous mutation in the *GABRB1* gene shifted their behavior to show robust and selective preference for ethanol in a two-bottle choice procedure. In operant self-administration procedures, the induced mutant self-administered ethanol to a greater extent (i.e., higher ethanol intakes,

greater number of active lever presses) than the wild types, especially at the higher ethanol concentrations (Anstee et al. 2013). In contrast, β2 knockout mice have been evaluated in a two-bottle choice procedure, and no differences in ethanol consumption or preference were found between the mutants and the wild types (Blednov et al. 2003). Although β3 knockout mice have been developed, they exhibit severe developmental problems and have not been used widely in ethanol-related studies (e.g., Homanics et al. 1997a). Much remains equivocal or unknown regarding the role of β subunits in the reinforcing effects of ethanol; however, the recent development of compounds with some selectivity for β subunits (e.g., etomidate) may offer one avenue for future investigations.

Finally, a significant association was found in the Plains Indian population between AUD and *GABRG1*, the gene encoding the γ1 subunit (Enoch et al. 2009). The potential importance of this subunit is underscored by a pilot study in humans that also found a relationship between a *GABRG1* polymorphism and IV ethanol self-administration in human volunteers (Plawecki et al. 2013). The results showed that individuals heterozygous at the polymorphism were significantly more motivated to self-administer than the homozygous individuals. Unfortunately, no γ1 transgenic mouse models have been developed. Moreover, although γ2 transgenic mouse models have been developed and have been exposed to ethanol, it has not been in the context of reinforcing effects. Finally, although there is evidence of haplotype and single nucleotide polymorphism association between alcohol dependence and *GABRG3* encoding the γ3 subunit (Dick et al. 2004), no γ3 mutant mouse models exist to explore the association. To our knowledge, no pharmacological tools exist to probe the role of the γ subunit in ethanol's behavioral effects.

4 Subjective Effects of Ethanol

4.1 Models of the Subjective Effects of Ethanol

The ability of ethanol to engender characteristic subjective effects may contribute importantly to its abuse. It has been suggested that the subjective effects of drugs may contribute to the initiation of drug taking in intermittent users and to the relapse process in drug abusers (Stolerman 1992). Evaluation of the subjective effects of ethanol is well established in clinical research, employing a sophisticated set of questionnaires (Kelly et al. 2003). Some of the most commonly used scales to study the subjective effects of ethanol include the Visual Analog Drug Effect Questionnaire (VAS), the Biphasic Alcohol Effect Scale, the Subjective High Assessment Scale, and the Alcohol Sensation Scale. The VAS questionnaire includes questions regarding whether the subject perceives they feel good/bad after the drinks, whether the effects of the drink are liked/disliked, and if they feel best/worst. Subjects rate the intensity of the effects on a scale of 1–100; items are then summed into positive (i.e., good/like/best) or negative (i.e., bad/dislike/worst) effects. The Biphasic Alcohol Effect Scale comprises 14 items assessing the sedative and stimulant effects of ethanol; subjects rate the perceived effects from 1 to 9. Measures again are grouped into stimulant and sedative categories. The Subjective High Assessment

Scale includes descriptions of the effects of ethanol (e.g., high, clumsy, dizzy, nauseated) and assesses the current perceived state in a continuum from "not at all" to "extremely." Finally, the Alcohol Sensation Scale measures somatic sensations elicited by ethanol which can be divided into six subscales: (1) central stimulant (effects on the brain), (2) dynamic peripheral (excitation, including breathing and heart rate), (3) warmth glow (blushing), (4) anesthetic (decreased or loss of feeling sensation), (5) gastrointestinal and (6) impaired function (changes in psychomotor execution).

Drug discrimination procedures provide a useful experimental counterpart to subjective effects because there is a close correspondence between the classification of drugs based on their discriminative stimulus effects in laboratory settings and subjective effects in humans (Schuster and Johanson 1988). They also serve as a valuable in vivo technique for evaluating underlying pharmacological mechanisms (Grant 1999). These procedures assess if a test drug produces similar interoceptive effects as a training drug. In drug discrimination, a subject is trained to differentiate between at least two conditions (e.g., training dose v. vehicle) based solely on interoceptive cues. During training, each condition is paired with a specific response (e.g., response on specific lever), and each correct response is reinforced (e.g., money in clinical studies or food reward in preclinical studies). Incorrect responses yield no reward. An accuracy criterion is typically set (e.g., at least 80% correct responses based on condition) and must be achieved before testing can begin. During testing, in which the discriminative stimulus effects of different doses of the training drug, novel drugs, or combinations can be investigated, all responses are rewarded as there is no correct or incorrect response (McMahon 2015; Bolin et al. 2016). Drug discrimination procedures are used in both clinical and preclinical settings.

4.2 $GABA_A$ Receptors and Subjective/Discriminative Stimulus Effects of Ethanol

4.2.1 α1-Containing $GABA_A$ Receptors

Clinically, one of the only α1 compounds available for evaluation in humans is the agonist zolpidem. In a study which evaluated subjective effects in healthy volunteers, both zolpidem and ethanol increased "drug-liking" and "drug strength perception" compared to placebo on the VAS scale when administered separately. However, when zolpidem was administered in conjunction with an ethanol-containing beverage, ethanol did not have additive effects on the subjective ratings for zolpidem. This lack of interaction suggests the lack of a common mechanism underlying the subjective effects of zolpidem and ethanol (Wilkinson 1998).

Preclinical studies using drug discrimination procedures also suggest an incomplete overlap of the discriminative stimulus effects of ethanol and α1 agonists. For example, the α1-preferring agonists zolpidem, abecarnil, and zaleplon only partially reproduced the ethanol discriminative stimulus up to doses that markedly reduced rates of responding in rats trained to discriminate ethanol from vehicle

(Bienkowski et al. 1997; Sanger 1997). These results suggest that stimulation of α1-containing $GABA_A$ receptors alone is not sufficient to produce ethanol-like discriminative stimulus effects.

In squirrel monkeys trained to discriminate ethanol from vehicle, zolpidem, zaleplon, and the α1-preferring partial agonist CL 218,872 engendered dose-dependent increases in ethanol-lever responding, but only zaleplon engendered ≥80% ethanol-lever responding (Platt et al. 2005). Importantly, in antagonism studies, the α1-preferring antagonist βCCt failed to alter the ethanol-like effects of zaleplon and zolpidem, or the effects of ethanol itself. These findings suggest that the α1 subunit plays little role in the discriminative stimulus effects of ethanol and, further, that the partial to full substitution profiles of α1 agonists are likely due to the binding of these drugs to other $GABA_A$ receptor subtypes that may have more prevalent roles in ethanol's subjective effects. An important caveat to this conclusion comes from a discrimination study in cynomolgus monkeys trained to discriminate either 1 or 2 g/kg ethanol from vehicle (Helms et al. 2008). In this study, zolpidem was found to fully substitute for the higher dose of ethanol in the majority of monkeys in this training group; whereas it fully substituted for the lower dose of ethanol in less than half of the monkeys in this training group. This finding suggests that an important determinant of ethanol's discriminative stimulus effects is the training dose and that different mechanisms may underlie the stimulus effects of different doses.

4.2.2 α2/3-Containing $GABA_A$ Receptors

Genetic association studies have identified single nucleotide polymorphisms in the *GABRA2* gene that appear to moderate expression of the subjective effects of ethanol (Covault et al. 2004). For example, homozygous carriers of the A-allele of the rs279858 polymorphism experience more intense subjective effects of ethanol than do homozygous or heterozygous carriers of the G-allele that previously has been associated with alcohol dependence (Pierucci-Lagha et al. 2005). These findings have been confirmed in several studies in which the same polymorphism and additional *GABRA2* polymorphisms were under study (e.g., rs279869, rs279837; Roh et al. 2011; Uhart et al. 2013). Moreover, Uhart et al. (2013) showed that carriers of the minor alleles that have been previously associated with alcoholism showed lower ethanol-induced "negative" subjective effects. As alcoholism risk often has been associated with an overall low level of response to ethanol, these findings make intuitive sense. To our knowledge, no functionally relevant polymorphisms that have been identified in the *GABRA3* gene have been evaluated in the context of ethanol's abuse-related effects. Moreover, no preclinical studies exist that explicitly evaluate the role of the α2 and/or α3 subunit in the discriminative stimulus of ethanol.

4.2.3 α5-Containing $GABA_A$ Receptors

Despite a number of α5 compounds available for preclinical study, only one inverse agonist (α5IA) has undergone sufficient toxicological testing that allows it to be studied in humans (Nutt et al. 2007). In a double-blind, placebo-controlled

crossover study, healthy volunteers were administered either α5IA or placebo, followed later by ethanol. Subjective effects were determined with the Subjective High Assessment Questionnaire, the Biphasic Alcohol Effects Scale, and the Alcohol Urge Questionnaire. The results showed that after treatment with the inverse agonist, the subjective effects of ethanol were unchanged.

The negative findings in humans are in direct contrast to preclinical pharmacological studies. For example, several studies have evaluated the ability of the α5-preferring inverse agonist Ro 15-4513 to attenuate the discriminative stimulus effects of ethanol. In male CD-1 mice trained to discriminate either ethanol from saline, Ro 15-4513 robustly attenuated ethanol's discriminative stimulus effects, engendering dose-dependent reductions in ethanol-lever responding (Rees and Balster 1988). Similar, although less robust, effects were observed in female C57BL/6 mice (Middaugh et al. 1991). In male rats, Ro 15-4513 has been shown to effectively block the effects of low-to-moderate ethanol doses without altering response rates (Gatto and Grant 1997), an effect not observed in female rats (Hilturnen and Järbe 1988), suggesting a potential sexually dimorphic effect of the drug in this species. In cynomolgus monkeys trained to discriminate ethanol from vehicle, pretreatment with Ro 15-4513 shifted the dose-response function rightward in all monkeys (Helms et al. 2009).

Using other selective pharmacological tools, the role of the α5 subunit was probed in ethanol-trained squirrel monkeys (Platt et al. 2005). In monkeys, the α5-preferring agonist QH-ii-066 and the selective partial agonist panadiplon dose-dependently engendered ethanol-like discriminative stimulus effects without significantly altering response rates. Moreover, the ethanol-like stimulus effects of QH-ii-066 were attenuated with the selective inverse agonists L-655,708 and RY023. Finally, the discriminative stimulus effects of ethanol itself were blocked by L-655,708. This study provides strong evidence of a key role for the α5 subunit in the subjective effects of ethanol. The reasons underlying the discrepancy between the human and monkey study are not entirely clear but could be related to the relative inverse efficacies of the compounds under study. Whereas α5IA has −25% efficacy at α5-containing receptors (Sternfeld et al. 2004), RY023 and L-655,708 at least have efficacies of −55% and −35%, respectively, at this subtype (June et al. 2001; Atack et al. 2006). It is possible that there is a threshold level of inverse efficacy that must be achieved to attenuate the behavioral effects of ethanol.

4.2.4 α4/6δ-Containing $GABA_A$ Receptors

A useful literature to examine to determine the role of the α4, α6, and/or δ subunits in the discriminative stimulus or subjective effects of ethanol is that related to neuroactive steroid (i.e., neurosteroid) regulation of the behavioral effects of ethanol. Neurosteroids are endogenous compounds whose actions are mediated in part by α4δ-containing receptors (Lambert et al. 2003). As reviewed in Helms et al. (2012), neurosteroids that positively modulate the $GABA_A$ receptor (e.g., allopregnanolone, pregnanolone) typically engender ethanol-like discriminative stimulus effects in laboratory animals, whereas those that negatively modulate the receptor (e.g., epipregnanolone, epiallopregnanolone) can reduce ethanol's

discriminative stimulus effects. Likewise, in pregnanolone-trained rats, ethanol has been found to partially substitute for the discriminative stimulus effects of the neurosteroid (Eppolito et al. 2012). Interestingly, though, δ subunit knockout mice learn to discriminate ethanol and show a similar profile of neurosteroid substitution as the wild types, indicating that the δ subunit is not critical for the ethanol-like stimulus effects of neurosteroids (Shannon et al. 2004).

Given the findings discussed above, one would predict a key role for the α4 subunit in the discriminative stimulus effects of ethanol. However, in animals (cynomolgus monkeys, mice) trained to discriminate ethanol, the α4δ-preferring drug THIP does not produce ethanol-like effects (Helms et al. 2012). Similarly, in rats trained to discriminate THIP, ethanol failed to engender any substitution up to doses that virtually eliminated responding nor did ethanol enhance the discriminative stimulus effects of THIP (Zanettini et al. 2016). These results suggest no overlap in the discriminative stimulus effects of the compounds. One possible explanation for these latter observations is that THIP is a direct GABA agonist and it appears to be the case that the shared effects of ethanol and neurosteroids occur at low-to-moderate doses that are likely to be modulatory rather than direct-acting at the $GABA_A$ receptor.

5 Relapse-Inducing Effects of Ethanol

5.1 Models of Ethanol Relapse

Relapse to alcohol consumption after a period of abstinence often occurs despite treatment, with 60–80% of abstinent alcoholics relapsing during their lifetime (Barrick and Connors 2002; Jaffe 2002). While several criteria for relapse are recognized, a defining criterion is a return to levels of ethanol consumption equal to or greater than that observed prior to abstinence. The alcohol deprivation effect (ADE) models relapse-like drinking and has been observed in both laboratory animals and humans (Burish et al. 1981; Vengeliene et al. 2014). The ADE can be defined as a temporary increase in ethanol intake over water upon reexposure to ethanol access compared with levels observed prior to a period of abstinence (for review see Vengeliene et al. 2014; Bell et al. 2017).

Relapse is often precipitated by stimuli that elicit craving for ethanol, including environmental stimuli associated with previous ethanol use (cues; Sinha and Li 2007), acute reexposure to ethanol (priming; Bensançon 1993; Sinha and O'Malley 1999), and stress (Sinha and Li 2007). Preclinically, the reinstatement procedure models alcohol seeking induced by these triggers. Animals initially are trained to self-administer ethanol, frequently under conditions in which ethanol delivery is paired with one or more environmental cues (e.g., lights, tones) or a particular environment. Operant behavior is then extinguished by omitting both ethanol deliveries and ethanol-paired cues. Testing consists of reexposure to ethanol, ethanol-paired cues, and/or a stressor (e.g., foot shock, yohimbine) in the absence of the ability to self-administer ethanol. Measures of ethanol-seeking behavior

include both rate of extinction (with slower rates presumed to reflect seeking behavior) and response rates after exposure to a relapse trigger (Le and Shaham 2002; Bossert et al. 2013). To date, there are very few studies reporting on the role of specific $GABA_A$ receptor subunits in any relapse model, let alone in relapse in humans.

5.2 $GABA_A$ Receptors and the Relapse-Inducing Effects of Ethanol

5.2.1 α1-Containing $GABA_A$ Receptors

As previously discussed, one α1-preferring antagonist (3-PBC) appears to increase the latency to initiate drinking in nonhuman primates (Kaminski et al. 2012; Sawyer et al. 2014) raising the possibility that this subunit plays a role in initiating drinking behavior after, perhaps, abstinence. However, the chained schedule under which baboons self-administered ethanol included a "link" that directly measured ethanol-seeking behavior, and measures associated with this link were unchanged by 3-PBC (Kaminski et al. 2012). Together, these studies provide only weak evidence for a role of the α1 subunit in ethanol seeking.

5.2.2 α2/3-Containing $GABA_A$ Receptors

To our knowledge, no preclinical studies have assessed the role of these subunits in relapse or relapse-related behaviors. However, a single clinical study (Kareken et al. 2010) assessed the effects of a polymorphism in the *GABRA2* gene on measures of craving and fMRI response to alcohol cues. Interestingly, all participants reported increased craving after exposure to alcohol odors/visual cues, with no genotype-dependent differences in intensity. The only difference between genotypes was in BOLD response to alcohol odors in medial frontal areas. These findings suggest little role for the α2 subunit in self-reported craving, as well as a dissociation between self-reports and a brain region previously associated with response to alcohol cues.

5.2.3 α5-Containing $GABA_A$ Receptors

In one study in which an α5-selective inverse agonist was administered to healthy human volunteers (Nutt et al. 2007), the drug failed to alter the subjective "urge to drink." As of now, no preclinical studies have assessed the effects of α5 drugs in relapse models, but these studies may be warranted given the positive effects of these ligands against other abuse-related effects of ethanol.

5.2.4 α4/6δ-Containing $GABA_A$ Receptors

As with other abuse-related effects of ethanol, information regarding α4/6-δ-containing receptors in ethanol relapse comes from studies with neurosteroids. In that regard, priming injections of allopregnanolone or the longer-acting synthetic neurosteroid ganaxolone induce ethanol-seeking behavior in reinstatement paradigms in rodents (Nie and Janak 2003; Finn et al. 2008; Ramaker et al.

2014) implying a specific role for these receptor subtypes in priming-induced reinstatement. Interestingly, though, THIP failed to engender ethanol-seeking behavior (Ramaker et al. 2014), again suggesting that there are clear differences among ligands that directly activate the receptor v. modulate the receptor. Additionally, neurosteroid-induced "seeking" responses are not always specific to ethanol as is evident in mice in which allopregnanolone induced both ethanol- and sucrose-seeking behavior (Finn et al. 2008). These latter findings suggest that, at least in mice, allopregnanolone may facilitate appetitive behavior in general, rather than ethanol-seeking in particular.

6 Conclusions

Ethanol's reinforcing and subjective effects, as well as its ability to induce relapse, are powerful factors contributing to its widespread use and abuse. A significant mediator of these behavioral effects is the $GABA_A$ receptor system; however, the contribution of particular subunits to specific behavioral effects is less clear and remains an area of research focus. Investigators have utilized various genetic and/or pharmacological approaches in pursuit of this understanding. Although both genetic and pharmacological approaches have limitations (e.g., compensatory changes in subunits in response to gene knockout, incomplete knowledge of compound-specific pharmacokinetics and brain penetration, limited knowledge of subunit distributions across model species), one can reach reasonable conclusions regarding the role of specific subunits in the abuse-related effects of ethanol based on a preponderance of convergent evidence across both species and assessment techniques.

As indicated, there exists a clear disparity in knowledge regarding the role of $GABA_A$ subunits, ranging from well-studied to virtually unstudied, depending on the behavioral effect. By far the most widely studied abuse-related effect of ethanol, in the context of $GABA_A$ subunits, is its reinforcing effects with both preclinical and genetic studies contributing to our knowledge base. Based on these studies, a key role has been suggested for the α5 subunit in ethanol reinforcement. Roles for the α1, α2, and α4/6δ subunits are more equivocal, and α3, β, and γ subunits remain understudied. Less well-studied in the context of $GABA_A$ mechanisms are the subjective/discriminative stimulus effects of ethanol. The strongest evidence of a role for a particular $GABA_A$ subunit in these effects is the clinical and genetic findings supporting a role for the α2 subunit (preclinical studies are lacking). Substantial preclinical evidence also implicates α5 subunits in the discriminative stimulus effects of ethanol. Roles for the α1 and α4/6δ subunits are more ambiguous, and the other subunits remain unstudied. As far as the relapse-inducing effects of ethanol, it is fair to say that there is no clear understanding of whether a particular $GABA_A$ subunit is important. There are simply too few clinical or preclinical studies that have addressed this question.

Acknowledgments The writing of this chapter was supported by NIH AA016179.

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Presynaptic Ethanol Actions: Potential Roles in Ethanol Seeking

David M. Lovinger

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Abstract

Ethanol produces intoxication through actions on numerous molecular and cellular targets. Adaptations involving these and other targets contribute to chronic drug actions that underlie continued and problematic drinking. Among the mechanisms involved in these ethanol actions are alterations in presynaptic mechanisms of synaptic transmission, including presynaptic protein function and excitation-secretion coupling. At synapses in the central nervous system (CNS), excitation-secretion coupling involves ion channel activation followed by vesicle fusion and neurotransmitter release. These mechanisms are altered by presynaptic neurotransmitter receptors and prominently by G protein-coupled receptors (GPCRs). Studies over the last 20–25 years have revealed that acute ethanol exposure alters neurotransmitter secretion, with especially robust effects on synapses that use the neurotransmitter gamma-aminobutyric acid (GABA).

The original version of this chapter was revised. A correction to this chapter is available at https://doi.org/10.1007/164_2018_189.

D.M. Lovinger (✉)
Laboratory for Integrative Neuroscience, Division of Intramural Clinical and Biological Research, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA
e-mail: lovindav@mail.nih.gov

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_76

Intracellular signaling pathways involving second messengers such as cyclic AMP and calcium are implicated in these acute ethanol actions. Ethanol-induced release of neuropeptides and small molecule neurotransmitters that act on presynaptic GPCRs also contribute to presynaptic potentiation at synapses in the amygdala and hippocampus and inhibition of GABA release in the striatum. Prolonged exposure to ethanol alters neurotransmitter release at many CNS GABAergic and glutamatergic synapses, and changes in GPCR function are implicated in many of these neuroadaptations. These presynaptic neuroadaptations appear to involve compensation for acute drug effects at some synapses, but "allostatic" effects that result in long-term resetting of synaptic efficacy occur at others. Current investigations are determining how presynaptic neuroadaptations contribute to behavioral changes at different stages of alcohol drinking, with increasing focus on circuit adaptations underlying these behaviors. This chapter will discuss the acute and chronic presynaptic effects of ethanol in the CNS, as well as some of the consequences of these effects in amygdala and corticostriatal circuits that are related to excessive seeking/drinking and ethanol abuse.

Keywords
Addiction · Alcohol · Amygdala · Cortex · Endocannabinoid · GABA · Glutamate · Long-term depression · Striatum · Synaptic plasticity · Synaptic transmission

1 Excitation-Secretion Coupling and Modulation at CNS Synapses

Communication between neurons generally occurs at synapses in which neurotransmitters are stored in and released from vesicles. When a neuronal action potential reaches the presynaptic terminal, the depolarization activates voltage-gated calcium channels (VGCCs) that allow calcium to enter the terminal. The increased intraterminal calcium stimulates vesicle fusion in a process known as excitation-secretion coupling (Catterall and Few 2008). Low rates of vesicle fusion also occur in the absence of excitation-secretion coupling, and this fusion appears to involve vesicle- and plasma membrane-associated proteins (Kavalali 2015). Upon release, the neurotransmitter is available to bind to receptor proteins that either directly gate ion flux (ligand-gated ion channels, LGICs) or act through intracellular GTP-/GDP-binding proteins (G proteins) that alter signaling processes (G protein-coupled receptors, GPCRs) (Betke et al. 2012; Latek et al. 2012).

While postsynaptic receptors are well known to transduce the signals necessary for anterograde transmission, presynaptic receptors have important roles in feedback alterations in the released neurotransmitter (via "autoreceptors") or crosstalk to alter release of other neurotransmitters (via "heteroreceptors"). Both LGICs and GPCRs serve as presynaptic receptors. The LGICs directly influence excitability of terminals (Engelman and MacDermott 2004; Pinheiro and Mulle 2008), and although these effects are interesting, they will not be considered in any detail in the remainder of this chapter given the relative lack of information on ethanol interaction with these presynaptic receptors. The presynaptic GPCRs work through a

variety of heterotrimeric G proteins and signaling pathways. The heterotrimeric G proteins consist of obligate α, β, and γ subunits, with the latter forming stable β/γ complexes. The G proteins are generally classified according to the type of α subunit present in the complex, and there are several major α subtypes. In this review the focus will be on three subtypes, Gαi/o, Gαq, and Gαs/olf. Upon GPCR activation the heterotrimeric complex separates into free α and β/γ components that then bind to intracellular signaling proteins to alter many aspects of cell biochemistry, gene expression, and physiology (Oldham and Hamm 2006).

Activation of GPCRs that couple to Gi/o generally inhibits excitation-secretion coupling and vesicle fusion and hence neurotransmitter release (Atwood et al. 2014; Miller 1998). The predominant mechanism involved in this modulation is inhibition of the VGCCs that mediate excitation-secretion coupling (Herlitze et al. 1996; Ikeda 1996). However, there is also strong evidence for direct G-protein inhibition of vesicle release (Blackmer et al. 2001). It must be emphasized that the Gβ/γ subunit produces these actions by direct binding to channels and vesicle-associated proteins. The Gαi/o subunit inhibits adenylyl cyclase (AC) and thus reduces intracellular cyclic AMP levels (Oldham and Hamm 2006). Inhibition of this enzyme is also implicated in inhibition of neurotransmitter release, especially in long-lasting inhibition (Atwood et al. 2014; Seino and Shibasaki 2005).

A wide variety of Gi/o-coupled GPCRs exist, with a subtype for almost every major neurotransmitter and neuromodulator. Many of these receptors will be discussed throughout this review, with a strong emphasis on the type 1 cannabinoid receptor (CB1). The CB1 receptor is the target of Δ9-tetrahydrocannabinol, the major psychoactive ingredient in preparations of *Cannabis sativa*. This receptor is normally activated by the endocannabinoid (eCB) fatty acid derivatives produced by hydrolysis of arachidonoyl membrane lipids (namely, arachidonoyl ethanolamide, or AEA also known as anandamide, and 2-arachidonoylglycerol, or 2-AG) (Araque et al. 2017). Functions of the gamma-aminobutyric acid B ($GABA_B$) receptor and the metabotropic glutamate receptor type 2 (mGluR2) will also be discussed in some detail.

The GPCRs that couple to Gq-containing GPCRs activate the hydrolysis of membrane phospholipids by phospholipases, a mechanism activated by the Gαq subunit (Oldham and Hamm 2006). The best-known pathway is activation of phospholipase C to catalyze the generation of diacylglycerol (DAG) and inositol phosphates. Among the many responses to Gαq actions are neuronal excitation through inhibition of voltage-gated potassium channels and activation of transient receptor potential (TRP) channels. These effectors may contribute to increased VGCC activation and increased neurotransmitter release (reviewed in Brown and Sihra 2008). However, Gβ/γ liberated by dissociation of heterotrimeric Gαq-containing proteins can inhibit VGCCs and neurotransmitter release (Brown and Sihra 2008). In addition, the DAG liberated by PLC-mediated hydrolysis can be further metabolized to the eCB 2-AG.

Activation of Gαs/olf G proteins leads to stimulation of AC activity and cAMP production, leading to stimulation of protein kinase A (PKA) and the exchange protein activated by cAMP (EPAC) proteins. GPCRs that activate Gαs/olf regulate a diverse array of biochemical, protein trafficking, and genetic regulation pathways.

The direct physiological consequences of this signaling are not widely known, but it has generally been observed that activation of some Gαs/olf-coupled receptors stimulates neurotransmitter release (reviewed in Brown and Sihra 2008). Forskolin, an AC activator, also increases neurotransmitter release at a variety of synapses, via a mechanism that involves cAMP and PKA activation.

1.1 GPCRs, Heterotrimeric G Proteins, and Synaptic Plasticity

Activation of presynaptic Gi/o-coupled GPCRs can produce either short- or long-lasting decreases in neurotransmitter release (Atwood et al. 2014). Inhibition of VGCCs and vesicle fusion are generally responsible for the short-lasting effects (persisting for seconds-to-tens of seconds). The longer-lasting effects (termed long-term depression or LTD) persist at least for hours and generally for as long as the preparation survives. It is not entirely clear what mechanisms contribute to Gi/o-LTD, but it is most likely that these mechanisms take place within the presynaptic neuronal elements with the axon terminal being the most likely site of action. LTD is observed in slice preparations in which the presynaptic soma is not present (e.g., at glutamatergic synapses in striatum in slices in which axons have been severed, as in Yin et al. 2006). Within the axon terminal, inhibition of AC is a prominent mechanism implicated in Gi/o-LTD, but long-lasting inhibition of VGCCs may also contribute (Atwood et al. 2014; Pelkey et al. 2008). Inhibition of AC will inhibit the activity of PKA, and this mechanism may also contribute to LTD. One PKA substrate, the Rim1 protein, has been implicated in presynaptic LTD (Heifets and Castillo 2009; Grueter et al. 2010). This phosphoprotein is associated with vesicles and implicated in control of vesicle fusion. Thus, it is thought that reducing PKA-catalyzed phosphorylation of Rim1 leads to a decrease in rates of fusion and neurotransmitter release. Presynaptic protein synthesis via translation also appears to have a key role in some forms of presynaptic Gi/o-LTD (Yin et al. 2006; Younts et al. 2017). An elegant recent study indicates that presynaptic GABAergic terminals in the hippocampus contain ribosomal elements that can mediate protein translation, and this process appears to be necessary for the expression of Gi/o-LTD at these synapses (Younts et al. 2017).

Presynaptic long-term potentiation (LTP) appears to involve Gs/olf-mediated processes (Evans and Morgan 2003; Waltereit and Weller 2003). For example, increased cAMP and PKA activation are implicated in the increased glutamate release observed during LTP at mossy fiber-CA3 pyramidal neuron synapses in the hippocampus (reviewed in Evstratova and Tóth 2014).

2 Acute Ethanol Effects on Neurotransmitter Release

Ethanol acts through a variety of molecular targets to produce acute intoxication. The stages of intoxication range from euphoria, anxiolysis, and enhanced movement (which can be quite variable across individuals) to motor and cognitive impairment, sedation, anesthesia, coma, and even death from respiratory depression

(Abrahao et al. 2017; Mihic and Harris 2011). The blood and brain ethanol concentrations generally associated with these lower-dose effects range from 5 to 10 mM at the low end through 18 mM (the legal intoxication level in the USA) up to ~100 mM which is the lethal range for average non-tolerant humans. Thus, in understanding the molecular and cellular bases of intoxication, it is important to examine effects of these relevant concentrations. The behavioral manifestations of intoxication are driven by effects on neurons (and possibly glia) in a number of brain regions and circuits that control everything from reward and movement to respiratory control (Abrahao et al. 2017). Thus, there is a need to understand actions on different cells in different regions to gain a fuller picture of how intoxication develops. It is also becoming clear that ethanol alters neuronal and synaptic activity via different mechanisms at different sites within the brain, and thus the field can no longer assume that effects involving one molecular target in one brain region will necessarily generalize to other regions (chapters in this volume, including: Anderson et al. 2017; Cannady et al. 2017; Chandler et al. 2017; Coleman and Crews 2017; Cuzon Carlson 2017; Dopico et al. 2017; Finn and Jimenez 2017; Hopf and Mangieri 2017; Klenowski and Tapper 2017; N'Gouemo 2017; Roberto et al. 2017; Schreiber and Gilpin 2017; Siciliano et al. 2017).

In this chapter the focus is on presynaptic ethanol effects. While ethanol has clear actions on targets within the postsynaptic elements of neurons, including a number of ligand-gated ion channels and potassium channels, these effects will not be discussed at present. The reader is referred to recent reviews that cover these subjects in detail (Abrahao et al. 2017; Harris et al. 2008; Lovinger and Roberto 2013; Roberto and Varodayan 2017).

2.1 GABA

Ethanol has its clearest acute presynaptic effects at GABAergic synapses in many brain regions. Early neurochemical studies showed both inhibitory and stimulatory effects of acute ethanol on GABA release in synaptosomal and brain slice preparations (Howerton and Collins 1984; Strong and Wood 1984; Seilicovich et al. 1988). It is not clear what accounted for these different findings, but they may be due to differences in the methods for stimulating release (mostly assayed with stimulation of release by increasing extracellular potassium concentrations) or the brain regions examined (e.g., as in Peris et al. 1992). Electrophysiological studies beginning in the 1990s began to establish that ethanol potentiation of GABAergic transmission at intact synapses is one of the clearest acute effects of the drug (Wan et al. 1996; Weiner et al. 1997). However, it was often assumed these ethanol effects only involved changes in $GABA_A$ receptor function. The first clear evidence of increased GABA release within particular brain regions came from studies in which the ethanol-induced potentiation was accompanied by decreased paired-pulse facilitation, changes in the frequency of miniature synaptic events, and other signs of presynaptic facilitation (Ariwodola and Weiner 2004; Nie et al. 2004; Roberto et al. 2003). Such effects were first reported at synapses made by

GABAergic neurons in the hippocampus (Ariwodola and Weiner 2004; Sanna et al. 2004). Subsequently, similar effects have been observed in the basolateral amygdala, central amygdala, cerebellum, dorsal striatum, nucleus accumbens, spinal cord, and ventral tegmental area (Bajo et al. 2008; Criswell et al. 2008; Kelm et al. 2008; Richardson and Rossi 2017; Silberman et al. 2008; Talani and Lovinger 2015; Theile et al. 2008; Wilcox et al. 2014; Ziskind-Conhaim et al. 2003). Ethanol also enhances GABA release onto cerebellar Purkinje neurons, although this effect appears to be due mainly to increased firing of Golgi-type interneurons (Carta et al. 2004). Within the BLA ethanol potentiates GABAergic synapses, with presynaptic mechanisms involved at one population of synapses and adrenergic-dependent postsynaptic mechanisms at another synaptic population (Silberman et al. 2008, 2012). Evidence for ethanol potentiation of glycine release has also been observed (Richardson and Rossi 2017; Ziskind-Conhaim et al. 2003).

Interestingly, some of the earliest reports of the presynaptic GABA release-enhancing ethanol effects also noted that these effects could be reduced by activation of the Gi/o-coupled $GABA_B$-type GPCR (Fig. 1a) (Ariwodola and Weiner 2004; Wan et al. 1996). This finding provided one of the first clues about the signaling pathways implicated in ethanol potentiation of GABA release. Subsequent studies have implicated the cyclic adenosine monophosphate (cAMP) intracellular signaling pathway in this ethanol action (Fig. 1a). Inhibition of adenylyl cyclase (the enzyme that catalyzes cAMP formation) and protein kinase A (PKA, the cAMP-activated protein kinase) has been shown to prevent this ethanol potentiation (Zhu and Lovinger 2006; Kelm et al. 2008; Talani and Lovinger 2015). The actions of Gi/o-GPCRs that prevent ethanol potentiation likely involve AC inhibition, which is a common consequence of activation of such receptors. Indeed, different Gi/o-GPCRs have now been shown to have this ethanol-inhibiting action at GABAergic synapses in several brain regions (Fig. 1a) (Kelm et al. 2008; Roberto et al. 2010; Talani and Lovinger 2015). This raises the possibility that such receptors may be used to alter ethanol effects, and indeed there is evidence that CB1, $GABA_B$, and mGluR2 receptor-targeted ligands may be useful in this context (Agabio and Colombo 2014; Meinhardt et al. 2013; Pava and Woodward 2012).

Additional mechanisms may also be involved in the presynaptic GABA-enhancing ethanol action. Knocking out the protein kinase C epsilon (PKCε) isoform appears to prevent ethanol effects in the central amygdala (CeA) (Fig. 1a) (Bajo et al. 2008). There may also be a role for stimulation of intracellular calcium release that could enhance excitation/secretion coupling in the cerebellum and VTA (Fig. 1a) (Kelm et al. 2007; Theile et al. 2009), and P-/Q-type VGCCs appear to be involved in ethanol potentiation in the CeA (Fig. 1a) (Varodayan et al. 2017). At several synapses, ethanol has been shown to increase the frequency of action potential- and calcium-entry-independent miniature inhibitory postsynaptic currents (mIPSCs) (Hirono et al. 2009; Kelm et al. 2007; Roberto et al. 2003; Talani and Lovinger 2015; Theile et al. 2008; Zhu and Lovinger 2006), and thus mechanisms downstream of VGCC function are likely involved in this effect (Fig. 1a). The function of vesicle- and plasma membrane-associated proteins involved in fusion could be

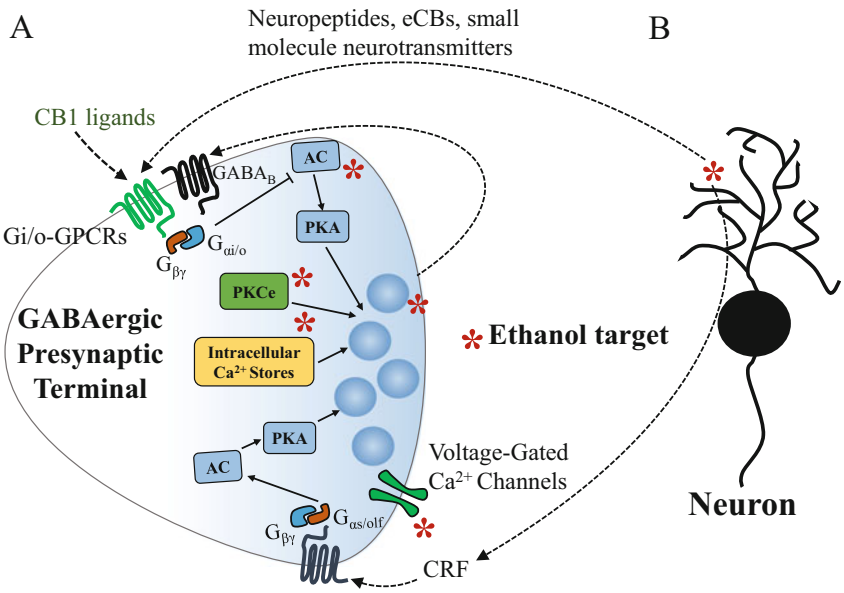

Fig. 1 Molecular targets and neuromodulators involved in acute presynaptic ethanol actions at GABAergic synapses. (**a**) Schematic diagram of a presynaptic terminal showing suspected sites of ethanol actions that enhance GABA release (asterisks). The main suspected targets are voltage-gated calcium channels, AC, vesicle fusion, PKCε, and intracellular Ca^{2+} stores. Neuropeptides, including CRF, eCBs, and small molecule neurotransmitters (including feedback vesicular GABA release), can contribute to or modulate ethanol actions on presynaptic GABA release through actions on presynaptic GPCRs. Note that ethanol enhances GABA release in many brain regions but inhibits release in others. (**b**) Ethanol is thought to stimulate release of neuropeptides (including enkephalins and CRF) and eCBs, presumably from neurons, and these neuromodulators act on presynaptic GPCRs to alter GABA release. Arrows indicate stimulation; cross-ended lines indicate inhibition. *AC* adenylyl cyclase, *CB1* cannabinoid type 1 receptor, *CRF* corticotrophin-releasing factor, *eCB* endocannabinoid, *GABA* gamma-aminobutyric acid, *GPCR* G protein-coupled receptor, *Gαi/o* alpha i/o G-protein subunit, *Gαs/olf* alpha s/olf subunit of G protein, *Gβγ* beta/gamma dimer subunit of G protein, *PKA* protein kinase A, *PKCε* protein kinase C epsilon

targets for ethanol actions, e.g., through changes in phosphorylation, but this has not yet been examined in detail.

The ethanol-induced increases in GABA release observed in brain slices could involve indirect effects due to release of neuromodulators that stimulate GABAergic terminals (Fig. 1a, b). In the CeA, ethanol potentiation of GABA release appears to involve activation of receptors for corticotrophin-releasing factor (CRF), presumably secondary to release of CRF itself (Fig. 1a, b) (Nie et al. 2004). Serotonin actions at the 5-HT2C receptor are implicated in ethanol potentiation in VTA (Theile et al. 2009). Application of the nociceptin peptide decreases GABA release in the CeA and prevents potentiation by ethanol when the peptide is applied before the drug (Roberto and Siggins 2006). Thus, increased release or decreased reuptake of small molecules or neuropeptides may underlie some of these ethanol actions.

However, experiments examining ethanol effects in an isolated "neuron-bouton" preparation provided evidence for a direct effect of ethanol on GABAergic presynaptic terminals (Zhu and Lovinger 2006; Kelm et al. 2007). These neurons are isolated mechanically such that pinched-off presynaptic boutons remain attached to the postsynaptic neuron. These boutons still release GABA, and thus spontaneous GABAergic IPSCs (sIPSCs) can be observed independent of the firing of GABAergic neurons and influences of any neurons other than the postsynaptic neuron (Jun et al. 2011). In this preparation, ethanol produces a rapid increase in the frequency of sIPSCs and mIPSCs, indicating a direct effect on GABAergic boutons that appears to be independent of known modulatory or retrograde signals form postsynaptic neurons (Zhu and Lovinger 2006).

It must also be noted that ethanol reduces GABAergic synaptic transmission at some CNS synapses. In the dorsolateral striatum (DLS), acute ethanol application produces such a reduction at synapses onto the medium spiny projection neurons (MSNs) made by both other MSNs and by parvalbumin-positive fast-spiking interneurons (FSIs) (Wilcox et al. 2014; Patton et al. 2016). The inhibition at FSI-MSN synapses appears to involve a presynaptic decrease in GABA release brought about through activation of delta opiate receptors (Fig. 1a) (Patton et al. 2016). This finding suggests increased production or release of yet another neuromodulatory peptide by acute ethanol, in this case an enkephalin (Fig. 1b). The emerging trend of ethanol modulatory effects through neuropeptide release opens up the possibility that the drug has a variety of actions at different synapses depending on the local peptide expression pattern.

Interactions at GABAergic synapses between the acute presynaptic effects of ethanol and endocannabinoids that act through the CB1 receptor have been especially noteworthy. Within the nervous system, eCBs are produced by postsynaptic elements in response to intense neuronal activity. These compounds travel retrogradely across the synaptic cleft to act on presynaptic CB1 receptors, Gi/o-GPCRs that inhibit neurotransmitter release (Fig. 1b). At synapses in the CeA and basolateral amygdala (BLA), CB1 activation prevents ethanol potentiation of GABA release (as described previously for other Gi/o-GPCRs) (Fig. 1a) (Kelm et al. 2008; Roberto et al. 2010; Talani and Lovinger 2015). There is also evidence that acute ethanol exposure can reduce retrograde eCB signaling at GABAergic synapses in the BLA (Talani and Lovinger 2015). In contrast, acute exposure to ethanol appears to enhance eCB-mediated LTD at glutamatergic synapses in the dorsomedial striatum (Yin et al. 2007). It is not yet clear what mechanisms account for the interaction of ethanol with eCB retrograde signaling. Interactions between ethanol and eCB/CB1 signaling may contribute to the alterations in the in vivo actions of ethanol produced by eCB-targeted drugs (Pava and Woodward 2012), a subject that will be discussed in greater detail in considering the effects of chronic ethanol exposure on the eCB signaling system.

2.2 Glutamate and Other Neurotransmitters

Acute ethanol exposure-induced alterations in glutamate release at CNS synapses have not been observed as frequently as effects on GABA release, but a few synapses show some sensitivity, with decreased release being the most common finding (Basavarajappa et al. 2008; Gioia and McCool 2017; Gioia et al. 2017; Li et al. 2013; Maldve et al. 2004; Silberman et al. 2015; Zhu et al. 2007). In the basolateral amygdala (BLA), ethanol inhibits glutamate release leading to decreased posttetanic potentiation, and reduced synaptic vesicle recycling appears to be the underlying mechanism (Gioia and McCool 2017). The vesicle-associated protein Munc13-2 is implicated in this effect (Gioia et al. 2017). Ethanol decreases glutamatergic synaptic transmission in CeA, and this effect is prevented by a CB1 agonist (Kirson et al. 2017) and may also involve N-type VGCCs (Zhu et al. 2007). While it is presumed that this effect involves presynaptic mechanisms, there is as yet no direct evidence that this is the case. Potentiation of glutamate release by ethanol has also been reported (e.g., Xiao et al. 2009; Deng et al. 2009) but less frequently than inhibitory actions.

The reasons for the differential effects of ethanol on GABA and glutamate release remain unclear. It is possible that presynaptic molecules that regulate intracellular calcium release and/or vesicle fusion differ at the different synaptic types. In addition, the effects on release secondary to increases in neuromodulator levels and subsequent activation of GPCRs may underlie these differential ethanol actions. This area should be a rich source of important new findings in the future.

There is evidence that presynaptic effects of ethanol alter release of other neurotransmitters, but in many cases, it is unclear if these effects involve direct drug actions on presynaptic terminals (Lovinger and Roberto 2013). In the striatum, ethanol inhibits DA release at relatively high concentrations in preparations where DAergic axon terminals are disconnected from their somata (Budygin et al. 2001). While this may still reflect an indirect modulatory action, the findings indicate a local effect on terminal DA release.

3 Presynaptic Neuroadaptations to Ethanol Exposure and Drinking

Prolonged exposure to ethanol, whether through forced exposure or ethanol drinking, produces neuroadaptations that often compensate for the acute drug actions. However, some adaptations are not always clearly compensatory, and sometimes appear to produce stable alterations that have an “allostatic” effect on neural function.

3.1 GABA

Both compensatory and allostatic neuroadaptations to ethanol have been observed at GABAergic synapses in different brain regions. While this chapter focuses on the presynaptic changes at GABAergic synapses, postsynaptic neuroadaptations have also been observed in many brain regions (e.g., Diaz et al. 2011; Abrahao et al. 2017; Roberto and Varodayan 2017). In the CeA, increased GABAergic transmission is observed following prolonged ethanol administration via vapor inhalation (Fig. 2) (Roberto et al. 2004a, 2010). While there is a prominent postsynaptic component to this neuroadaptation, there is also evidence that the probability of GABA release and/or the number of GABAergic synapses contributes to this effect. Reduced function of $GABA_B$ presynaptic autoreceptors is one factor that appears to

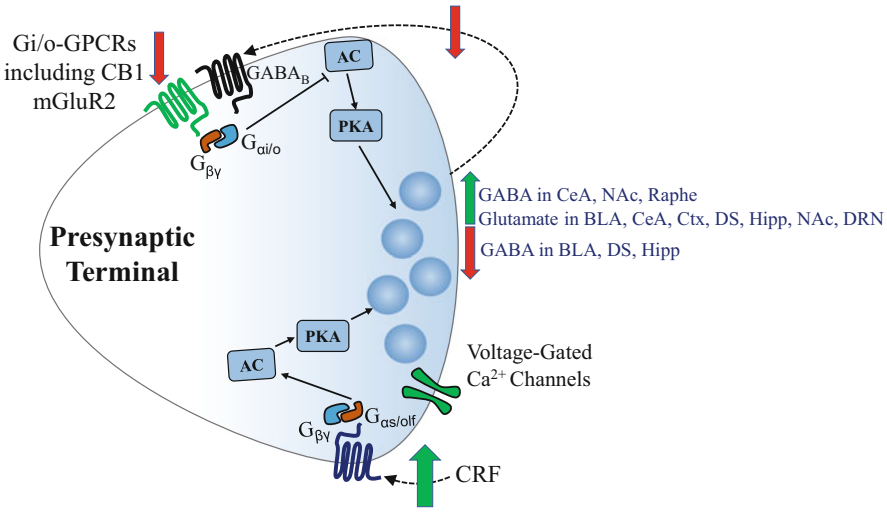

Fig. 2 Presynaptic neuroadaptations to chronic ethanol exposure/consumption. Green arrows indicate increases in GABA and glutamate release observed in several brain regions, as well as increased CRF that drives increased GABA release in CeA. Red arrows indicate decreases in Gi/o-GPCR expression/function that occur at both GABAergic and glutamatergic synapses (including decreased GABA/$GABA_B$R feedback onto GABAergic terminals), as well as decreased GABA release observed in some brain regions. Decreases in GABA release are thought to compensate for ethanol-induced increases in neurotransmitter release at GABAergic synapses, while activation of $GABA_B$ Gi/o-GPCRs participates in compensatory negative feedback that produces tolerance to the direct ethanol action on release (dashed arrow). At glutamatergic synapses, increased neurotransmitter release and decreased Gi/o-GPCR function may compensate for ethanol-induced decreases in glutamatergic transmission. Neuroadaptations that have a more allostatic role include increased GABA release and increased CRF signaling that enhances GABA release. *AC* adenylyl cyclase, *BLA* basolateral amygdala, *CB1* cannabinoid type 1 receptor, *CeA* central amygdala, *CRF* corticotrophin-releasing factor, *Ctx* cortex, *DS* dorsal striatum, *GABA* gamma-aminobutyric acid, $GABA_B$ GABA type B receptor, *GPCR* G protein-coupled receptor, *Gαi/o* alpha i/o G-protein subunit, *Gαs/olf* alpha s/olf subunit of G protein, *Gβγ* beta/gamma dimer subunit of G protein, *Hipp* hippocampus, *NAc* nucleus accumbens, *DRN* dorsal raphe nucleus, *PKA* protein kinase A

contribute to this increase in release (Fig. 2) (Roberto et al. 2008). Disrupted eCB/CB1 modulation of GABA release may also contribute to the increased release following chronic ethanol exposure (Varodayan et al. 2016). Alterations in CRF levels and function could well play a role in the chronic ethanol actions on CeA GABAergic transmission given the CRF potentiation involved in the acute drug action that was discussed previously (Figs. 1 and 2). Indeed, CRF levels are increased in the amygdala following withdrawal after chronic ethanol exposure, as measured with in vivo microdialysis (Merlo Pich et al. 1995). The ability of CRF to enhance GABA release in CeA is augmented in ethanol-dependent rats. The acute ethanol-induced potentiation of GABAergic transmission on CeA neurons remains intact following chronic exposure, indicating a lack of tolerance to ethanol. Withdrawal following chronic ethanol intake results in increased extracellular CRF levels in the BNST (Olive et al. 2002), but it is not clear if the increase alters GABAergic transmission in this region. Overall, GABAergic neuroadaptations in the CeA, and perhaps other parts of the extended amygdala, are not compensatory but rather induce a general enhancement of inhibition within CeA that is exacerbated during intoxication.

GABA release at hippocampal synapses may also be altered through changes in presynaptic function and modulation. In the dentate gyrus hippocampal subfield, there is evidence of decreased probability of GABA release following chronic ethanol intake in monkeys (Fig. 2) (Weiner et al. 2005). Evidence for decreased GABA release has also been observed in the CA1 subfield (Cagetti et al. 2003). These effects would appear to compensate for the increased GABA release during acute ethanol exposure. However, decreased $GABA_B$ receptor function has been implicated in increased GABA release in the CA1 subfield in vivo, and this may be an allostatic type of neuroadaptation (Peris et al. 1997). Decreased GABAergic transmission, involving both pre- and postsynaptic mechanisms has also been observed in the BLA following chronic ethanol drinking, and this neuroadaptation likely contributes to negative affective states that develop during withdrawal (Diaz et al. 2011).

GABAergic synaptic transmission onto serotonergic neurons in the dorsal raphe nucleus is not altered by acute ethanol exposure in naïve mice of the DBA1/J strain. However, following chronic ethanol exposure, acute application of the drug produces a robust enhancement of GABA release (Fig. 2) (Lowery-Gionta et al. 2015). This illustrates a case where a change in transmission does not directly compensate for an acute drug effect, but instead chronic exposure induces a hypersensitivity to ethanol that may alter the pattern of intoxication during subsequent encounters with the drug.

In the DLS, long-term changes at GABAergic synapses onto MSNs are mainly allostatic. Decreased frequency of GABAergic mIPSCs has been observed in both mouse DLS and the monkey putamen nucleus (roughly equivalent to rodent DLS) following chronic ethanol drinking protocols (Wilcox et al. 2014; Cuzon Carlson et al. 2011). These findings indicate that the effect of chronic ethanol exposure is similar to that of acute exposure, with the net effect being a loss of inhibition of MSN activity/striatal output (Fig. 2). In mouse DLS the effect of acute ethanol is

lost after chronic drinking (Wilcox et al. 2014), and thus the effect of chronic ethanol consumption sets a new level of GABAergic inhibition that appears to be stable.

Chronic ethanol drinking leads to depression of DMS GABAergic synapses, i.e., decreased mIPSC frequency, similar to that observed in DLS (Fig. 2) (Wilcox et al. 2014). This decrease is accompanied by a change from acute ethanol potentiation of GABA release to a slight depression. In the monkey caudate nucleus, GABAergic synaptic transmission exhibits smaller changes following chronic drinking than those observed in the putamen, but decreased mIPSC frequency is the most consistent observation (Cuzon Carlson et al. 2017). Thus, the general effect of ethanol on striatal GABAergic transmission is a decrease that would generally allow for increased striatal output driven by synaptic activation of MSNs.

In the VTA, a single in vivo ethanol exposure appears to produce increased GABA release at synapses on dopaminergic neurons (Melis et al. 2002; Wanat et al. 2009). This potentiation may involve impaired function of $GABA_B$ autoreceptors (Melis et al. 2002). However, effects of more prolonged ethanol exposure remain to be determined.

3.2 Glutamate and Dopamine

Prolonged ethanol exposure or drinking has generally been proposed to produce an increase in extracellular glutamate levels (Fig. 2) (Dahchour and De Witte 1999, 2003; Griffin et al. 2014; Meinhardt et al. 2013; Rossetti and Carboni 1995; Roberto et al. 2004b; Knackstedt and Kalivas 2009). The main evidence supporting this idea comes from microdialysis data demonstrating increases in extracellular glutamate in the cortex, dorsal striatum, NAc, and other brain regions (Dahchour and De Witte 1999, 2003; Knackstedt and Kalivas 2009; Meinhardt et al. 2013; Rossetti and Carboni 1995). However, it is not clear that the glutamate measured with this approach is of synaptic origin (e.g., Baker et al. 2002). Indeed, changes in the function of the cystine-glutamate transporter account for some of this increase (Baker et al. 2002; Knackstedt and Kalivas 2009). Nonetheless, there is evidence for presynaptic changes at glutamatergic synapses that would promote increased glutamate release and direct evidence for increased glutamate release at some brain synapses (Cuzon Carlson et al. 2011; Lack et al. 2007; Lowery-Gionta et al. 2015; Ma et al. 2017; Meinhardt et al. 2013; Zhu et al. 2007; Roberto et al. 2004b). There is also evidence for decreased glutamate uptake in the NAc following chronic ethanol drinking (Melendez et al. 2005). It should also be noted that synaptic glutamate release appears to be decreased in the lateral CeA following chronic ethanol exposure and a 48 h withdrawal (Pleil et al. 2015). Thus, with some exceptions, it appears that ethanol produces increased glutamate release at synapses in many brain regions.

Another synaptic change that appears to contribute to increased glutamate levels is the loss of regulation of release by presynaptic Gi/o-coupled receptors (Fig. 2). In the NAc and DS, mGluR2 acts presynaptically as an autoreceptor to reduce

glutamate release (Lovinger and McCool 1995; Manzoni et al. 1997). Chronic ethanol exposure decreases mGluR2 expression and function (Meinhardt et al. 2013), and this study supports the idea that loss of the mGluR2 autoreceptor function contributes to enhanced glutamate levels in NAc. Interestingly, mGluR2 is not expressed by the ethanol-preferring P rats and is missing in other rat lines selected for high ethanol drinking preference (Zhou et al. 2013; Wood et al. 2017). The impact of this receptor on ethanol seeking and drinking will be discussed later in this chapter.

Dopamine release in the nucleus accumbens is also altered following chronic ethanol exposure or drinking in rats and mice and in chronic ethanol-consuming rhesus monkeys (Siciliano et al. 2017). Some of these changes appear to reflect direct neuroadaptations in dopamine release mechanisms such as decreased release in brain slices (Karkhanis et al. 2015; Melchior and Jones 2017), while others indicate increased dopamine clearance, most likely due to changes in function of the dopamine transporter (Karkhanis et al. 2015, 2016). It is notable that dopamine release in male monkey NAc slices is increased following chronic drinking (Siciliano et al. 2015), in contrast to the findings in rodent. For a more in-depth discussion of these findings, the reader is referred to the excellent chapter by Siciliano et al. (2017) in this volume. Inhibition of dopamine release by the Gi/o-coupled kappa opioid receptor is also enhanced after chronic ethanol exposure in rodent NAc (Karkhanis et al. 2016; Rose et al. 2016), contributing to a possible hypodopaminergic state after this exposure. A similar enhancement of kappa receptor function is observed in NAc and caudate nucleus of chronic ethanol-consuming monkeys (Siciliano et al. 2015, 2016). Overall, several factors contribute to an overall decrease in synaptic dopamine levels following chronic ethanol exposure, particularly during the early stages of abstinence (Hirth et al. 2016). However, increased DA levels have been observed following protracted abstinence in rat, and molecular changes that could contribute to increased extracellular dopamine have been observed in postmortem tissue from patients with AUD (Hirth et al. 2016). These findings indicate that changes in factors controlling extracellular DA levels may depend on the period of drug withdrawal.

3.3 Endocannabinoids and LTD

The CB1 receptor is another presynaptic Gi/o-coupled GPCR whose function is decreased following long-term ethanol exposure (Fig. 2) (Xia et al. 2006; Adermark et al. 2011a, b; Depoy et al. 2013). As mentioned previously, retrograde signaling by postsynaptically released eCBs normally activates presynaptic CB1 receptors inducing either short-term synaptic depression or Gi/o-LTD at GABAergic and glutamatergic synapses throughout the brain (Araque et al. 2017; Heifets and Castillo 2009).

Chronic ethanol exposure or drinking produces decreased CB1 expression and function and loss of the LTD induced by activation of this receptor (Basavarajappa et al. 1998; Xia et al. 2006; Adermark et al. 2011a; DePoy et al. 2013). In the dorsal

striatum, depression at glutamatergic synapses induced by a CB1 agonist is lost following chronic ethanol drinking (Adermark et al. 2011a). The loss of CB1-mediated LTD persists for 7 days following the last drug exposure (Xia et al. 2006). At GABAergic synapses, eCB-dependent LTD also occurs, and this synaptic depression indirectly produces a long-lasting increase in neuronal activation by glutamatergic synapses (Adermark et al. 2009). This type of LTD is also impaired following chronic ethanol drinking, facilitating a long-lasting potentiation of striatal output in response to glutamatergic transmission (Adermark et al. 2011b). These changes in eCB-dependent plasticity combine with the decrease in GABAergic transmission to increase striatal output in ethanol-exposed animals.

4 Roles of Presynaptic Changes in Ethanol-Related Behaviors: Focus on Cortico-Basal Ganglia and Amygdala Circuitry

As the preceding sections indicate, we now know a great deal about the acute and chronic ethanol effects on neurotransmitter release as well as presynaptic modulation and plasticity. However, less is known about the roles played by these ethanol actions in the behavioral alterations induced by ethanol. Regarding the consequences of altered GABA release, there are well-known interactions between the acute effects of ethanol and many drugs that act at GABAergic synapses (Mihic and Harris 2011). However, these interactions have been mainly ascribed to ethanol effects on $GABA_A$ receptors. Thus, it will be important to investigate if presynaptic changes at GABAergic synapses contribute to the drug interactions. This is certainly an important topic, because ethanol drinking in conjunction with drugs that target GABAergic transmission can result in profound acute toxicity, including death.

There has been considerable recent attention on ethanol-induced alterations in presynaptic modulation at synapses in different regions of the striatum. This topic is of interest to investigators examining ethanol seeking and drinking because different cortico-basal ganglia circuits involving specific striatal subregions are implicated in these behaviors. Large regions of the striatum are part of at least three different cortico-basal ganglia circuits, with the DMS/caudate being part of an "associative" circuit, the DLS/putamen participating in the "sensorimotor" circuit, and the NAc being incorporated into the "limbic" circuit (Yin and Knowlton 2006). As discussed in the previous sections of this chapter, ethanol has effects on aspects of presynaptic function in striatal components of all these circuits.

The behavioral consequences of ethanol actions in NAc are widely appreciated (Koob and Volkow 2016). It is clear that this region and the associated limbic circuit have crucial roles in the rewarding effects of the drug (as shown using conditioned place preference and ethanol self-administration procedures). Indeed, alterations within the NAc/limbic circuit are likely to impact affective states, Pavlovian conditioning and Pavlovian-to-instrumental transfer conditioning, as well as responses to stress, withdrawal and other factors that contribute to negative

affect that helps to drive relapse to drinking. A decrease in mGluR2 modulation of glutamate release at prefrontal cortical inputs to the NAc contributes to excessive seeking and drinking following chronic ethanol exposure (Meinhardt et al. 2013). Rats and mice lacking mGluR2 also show enhanced ethanol seeking and drinking (Zhou et al. 2013; Wood et al. 2017), although it is clear that loss of mGluR2 is only one of several genetic and molecular factors that influence these behaviors in alcohol-preferring rats (Zhou et al. 2013). The consequences of mGluR2 absence or hypofunction presumably reflect loss of a crucial feedback control that normally prevents the hyperglutamatergic state thought to drive excessive drinking. Treatment with mGluR2/3 agonists reduces ethanol seeking in rodent models (Backstrom and Hyytia 2005; Rodd et al. 2006; Sidhpura et al. 2010; Zhao et al. 2006). However, considerable additional work is needed to determine the contributions of other presynaptic mechanisms in NAc (e.g., alterations in other presynaptic Gi/o-coupled GPCRs or altered GABA release) to ethanol actions in vivo.

Striatal function within the associative and sensorimotor circuits also has the potential to contribute to a variety of acute and chronic ethanol actions. The major focus of research in this area has been the dissociation of effects on "goal-directed" and "habitual" behaviors, including ethanol seeking and drinking (Lovinger and Alvarez 2017; Corbit and Janak 2016; Gremel and Lovinger 2017). Indeed, the DMS/caudate is implicated in goal-directed behaviors, while the DLS has a key role in habit learning, especially in self-paced "free-choice" instrumental tasks and response learning tasks. However, this facile dichotomy has overshadowed important roles of these regions and the larger circuits in behavioral control and ethanol actions.

For example, the associative striatum receives strong synaptic inputs from many regions of frontal cortex, including orbitofrontal and medial prefrontal areas (Haber et al. 2006; Hintiryan et al. 2016; Hunnicutt et al. 2016). These cortical regions show structural and functional alterations following long-term ethanol exposure, both in experimental animals and in humans (reviewed in Barker et al. 2015; Sullivan and Pfefferbaum 2005). The caudate nucleus also shows reduced volume after heavy drinking in adolescents (Squeglia et al. 2014). Thus, the associative circuit is likely to be strongly compromised by this type of ethanol exposure. Given the key role of the DMS/caudate within this circuitry, it is very likely that altered cortical communication to this striatal region contributes to this dysfunction. The evidence that acute and chronic ethanol produce presynaptic alterations in the DMS and caudate has already been discussed. The "hypofrontality" and altered DMS/caudate function induced by ethanol are likely to contribute to deficits in cognitive function and altered decision-making induced by ethanol abuse. The loss of conscious executive control during intoxication and following chronic ethanol abuse is likely to contribute to poor decision-making and preservation in drinking and other associated maladaptive behaviors. There is a growing literature showing that manipulation of the DMS alters ethanol seeking and drinking (Cheng et al. 2017; Corbit et al. 2012; Nam et al. 2013; Wang et al. 2012), but more work is needed to determine the mechanisms within this striatal region that contribute to this behavioral change.

The sensorimotor circuit has important roles in performance of well-learned actions. Inputs from sensory and motor cortices drive neurons in the DLS/putamen (Haber et al. 2006; Hintiryan et al. 2016; Hunnicutt et al. 2016) allowing for output of automatized movements in appropriate contexts. This circuitry also has key roles in reinforcement-driven "stimulus-response" learning and behavior, especially in self-paced operant tasks that do not include a clear Pavlovian component (Yin and Knowlton 2006). Repeated performance of actions for an outcome in a particular context leads to development of associations between the external context, the internal state of the animal, and the action, driven by the history of reinforcement (Dickinson 1985). Indeed, this form of instrumental learning, now sometimes referred to as "habit" learning, received the strongest attention prior to characterization of action-outcome "goal-directed" instrumental learning (Colwill and Rescorla 1990; Dickinson 1985). A number of studies have now shown that chronic ethanol drinking or exposure enhances this type of behavior in both experimental animals and humans (Barker et al. 2010; Corbit et al. 2012; Dickinson et al. 2002; Gladwin and Wiers 2012; Hogarth et al. 2012; Ostlund et al. 2010; Hay et al. 2013; Mangieri et al. 2012; Sjoerds et al. 2013 although see Sebold et al. 2014, 2017), as well as other behaviors that involve the DLS (DePoy et al. 2013).

A number of presynaptic changes in the DLS/putamen have been discussed, including decreased GABA release and decreased Gi/o modulation of cortical/glutamatergic inputs to this striatal subregion. The general consequence of these alterations is to decrease modulatory and inhibitory controls on the activation of MSNs, producing the potential for enhanced DLS/putamen output after chronic ethanol exposure. This would help to foster the learning and performance mediated by the sensorimotor circuit, including increased S-R learning. It remains to be determined what other presynaptic changes occur in other parts of the circuitry that could also contribute to these behavioral changes.

While the focus of the work on ethanol and sensorimotor circuitry has been on how enhanced "habit formation" might contribute to ethanol seeking and drinking, it is important not to lose sight of how the drug effects on sensorimotor circuitry will alter all behaviors related to this circuitry. For example, the fact that ethanol increases S-R learning reinforced by food is part of the pattern of impaired decision-making produced by the drug. This effect has consequences across the entire spectrum of behaviors altered by ethanol abuse. In combination with impairment of associative circuit function, enhanced potential for sensorimotor circuit function likely contributes to loss of executive control and behavioral flexibility with enhanced control of behavior by the immediate context. It is worth noting that this effect does not depend on having ethanol as the reinforcer driving learning, as S-R learning is enhanced by forced ethanol exposure when a food reinforcer is used in training (Corbit et al. 2012). Thus, it is unlikely that the enhanced S-R learning is driven by the reinforcement history per se. Rather, it appears to be the effect of ethanol is on the circuitry that influences how reinforcement drives behavior.

The implications of these presynaptic changes in particular circuit changes for ethanol seeking and drinking and other drug-related behaviors can be debated, but

there is evidence from studies in both experimental animals and humans that they have important roles. Multiple circuits contribute to intoxication, binge and excessive ethanol drinking, withdrawal effects, relapse to drinking, and excessive drinking following relapse. Acute exposure to ethanol initiates the processes that contribute to escalation of drinking. Presynaptic inhibitory changes in the BLA, CeA, and VTA likely contribute to the rewarding effects of ethanol. Enhanced inhibition in the associative circuit may play a part in impairment of cognitive control and executive function that contributes to lack of ability to consciously control drinking as well as poor decisions made under the influence of ethanol. Disinhibition of sensorimotor striatum most likely fosters excessive drinking and poor decision-making by fostering more automatized action patterns. Chronic ethanol effects will exacerbate many of these changes, particularly the presynaptic effects on amygdala and sensorimotor circuitry. Presynaptic changes within the limbic circuitry may also begin to have a larger influence with increasing duration and amount of chronic ethanol exposure. Increased inhibition in the hippocampal CA1 region can impair spatial memory and other aspects of episodic learning and memory (Berry et al. 2009; Gibson 1985; Givens 1995; Hunt et al. 2009; Matthews et al. 2002; Ryabinin 1998; Ryabinin et al. 2002). Long-term effects of changes in this limbic region may also underlie the influence of context on relapse to ethanol seeking and taking. Clearly ethanol has strong effects on GABA release in the CeA, with CRF participating in both the acute and chronic drug actions. There is now considerable evidence for participation of these neurotransmitters, and this brain region in relapse driven by stress and negative affect (Koob and Volkow 2016). The BLA plays important roles in signaling the relative positive or negative valence of environmental events within the associative and limbic circuits (Johansen et al. 2011; Wassum and Izquierdo 2015). Presynaptic ethanol effects at both GABAergic and glutamatergic synapses likely alter the contribution of this brain region to reward- and punishment-driven behavior, as well as responses to stress. Presynaptic effects of ethanol that alter serotonergic neuronal function will also alter limbic circuit responses to stress, in addition to affecting affective states.

The alterations in all three cortico-basal ganglia circuits, including presynaptic changes, will ultimately participate in a vicious circle of behavioral changes similar to that proposed by Koob and others (Barker et al. 2015; Koob and Volkow 2016). Prolonged ethanol exposure combined with conditioning related to ethanol intake will promote loss of associative circuit-based mechanisms that normally support decisions to limit drinking. Ethanol will also promote transition from associative/limbic-based reward-driven actions to sensorimotor reinforcement-based actions that will promote excessive drinking, especially in environments previously associated with heavy drinking. This will drive further neuroadaptations including impairment in prefrontal cortex contributions to associative and limbic circuits leading to compromised executive control and conscious decision-making. Parallel changes in other limbic cortical areas will promote excessive responding to negative emotions and stressful/negative environmental events, especially during abstinence. These limbic changes will help to promote relapse. In the proper environmental/social contexts relapse will be fostered by a strengthened sensorimotor circuit, and once drinking has

begun, the dominant sensorimotor circuit and impaired associative circuit will likely contribute to continued drinking due to automatization of behavior. Often drinking will then proceed well beyond levels needed to simply overcome negative consequence of abstinence. It will be interesting to determine more about how components of each of these circuits contribute to different stages of alcohol abuse. For example, little is known about how acute and chronic ethanol exposure alters sensory and motor cortex function and how these actions might contribute to altered circuit function. Even less is known about ethanol actions on the thalamic elements of the three circuits or effects on basal ganglia regions downstream of the striatum. Presynaptic ethanol actions may occur in many of these brain regions, and discovery of these effects may add to the list of potential targets for treatment of alcohol use disorders.

Acknowledgements This work was supported by the Division of Intramural Clinical and Biological Research of the National Institute on Alcohol Abuse and Alcoholism, project ZIA AA000407.

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Dynamic Adaptation in Neurosteroid Networks in Response to Alcohol

Deborah A. Finn and Vanessa A. Jimenez

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Abstract

The term neurosteroid refers to rapid membrane actions of steroid hormones and their derivatives that can modulate physiological functions and behavior via their interactions with ligand-gated ion channels. This chapter will highlight recent advances pertaining to the modulatory effects of a select group of neurosteroids that are primarily potent positive allosteric modulators of γ-aminobutyric $acid_A$ receptors ($GABA_ARs$). Nanomolar concentrations of neurosteroids, which occur in vivo, potentiate phasic and tonic forms of $GABA_AR$-mediated inhibition,

D. A. Finn (✉)
Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA

VA Portland Health Care System, Portland, OR, USA
e-mail: finnd@ohsu.edu

V. A. Jimenez
Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA

Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Portland, OR, USA
e-mail: wakeling@ohsu.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_82

indicating that both synaptic and extrasynaptic $GABA_A$Rs possess sensitivity to neurosteroids and contribute to the overall ability of neurosteroids to modulate central nervous system excitability. Common effects of alcohol and neurosteroids at $GABA_A$Rs have stimulated research on the ability of neurosteroids to modulate alcohol's acute and chronic effects. Background on neurosteroid pharmacology and biosynthetic enzymes will be provided as it relates to experimental findings. Data will be summarized on alcohol and neurosteroid interactions across neuroanatomical regions and models of intoxication, consumption, dependence, and withdrawal. Evidence supports independent regulation of neurosteroid synthesis between periphery and brain as well as across brain regions following acute alcohol administration and during withdrawal. Local mechanisms for fine-tuning neuronal excitability via manipulation of neurosteroid synthesis exert predicted behavioral and electrophysiological responses on $GABA_A$R-mediated inhibition. Collectively, targeting neurosteroidogenesis may be a beneficial treatment strategy for alcohol use disorders.

Keywords
Allopregnanolone · Consumption · Ethanol · $GABA_A$ receptors · Withdrawal

1 Introduction

Steroid hormones and their derivatives can influence brain function and behavior via classical genomic actions and rapid membrane effects (see Fig. 1 for biosynthetic pathway). Pioneering studies of Hans Selye (1942) reported the sedative-anesthetic activity of several steroidal compounds. Seminal studies by Margarethe Holzbauer and her colleagues isolated and identified many steroidal compounds from the ovarian venous blood of the rat (reviewed by Holzbauer 1976) and demonstrated the in vivo secretion of pregnenolone, progesterone, and allopregnanolone (ALLO; 3α,5α-THP or tetrahydroprogesterone) by the adrenal gland of the rat in quantities similar to those secreted by the ovary in estrus (Holzbauer et al. 1985). Then, a mechanism underlying rapid steroid actions was provided by the demonstration that the synthetic steroid alphaxalone potentiated γ-aminobutyric $acid_A$ receptor ($GABA_A$R)-mediated chloride currents (Harrison and Simmonds 1984). Subsequently, evidence accumulated that alphaxalone and steroid derivatives have rapid membrane actions via an interaction with ligand-gated ion channels (e.g., Belelli and Lambert 2005; Belelli et al. 1990; Paul and Purdy 1992; Purdy et al. 1990; Rupprecht and Holsboer 1999; Veleiro and Burton 2009). These findings gave rise to the terms "neuroactive steroids" and "neurosteroids" to refer to the rapid membrane actions and prompted interest in the ability of endogenous neurosteroids to modulate physiological functions and behavior (e.g., Belelli and Lambert 2005; Finn and Purdy 2007; Porcu et al. 2016; Zorumski et al. 2013).

Alcohol administration affects multiple neurotransmitter systems, and common effects of alcohol and neurosteroids at $GABA_A$Rs have stimulated research on the

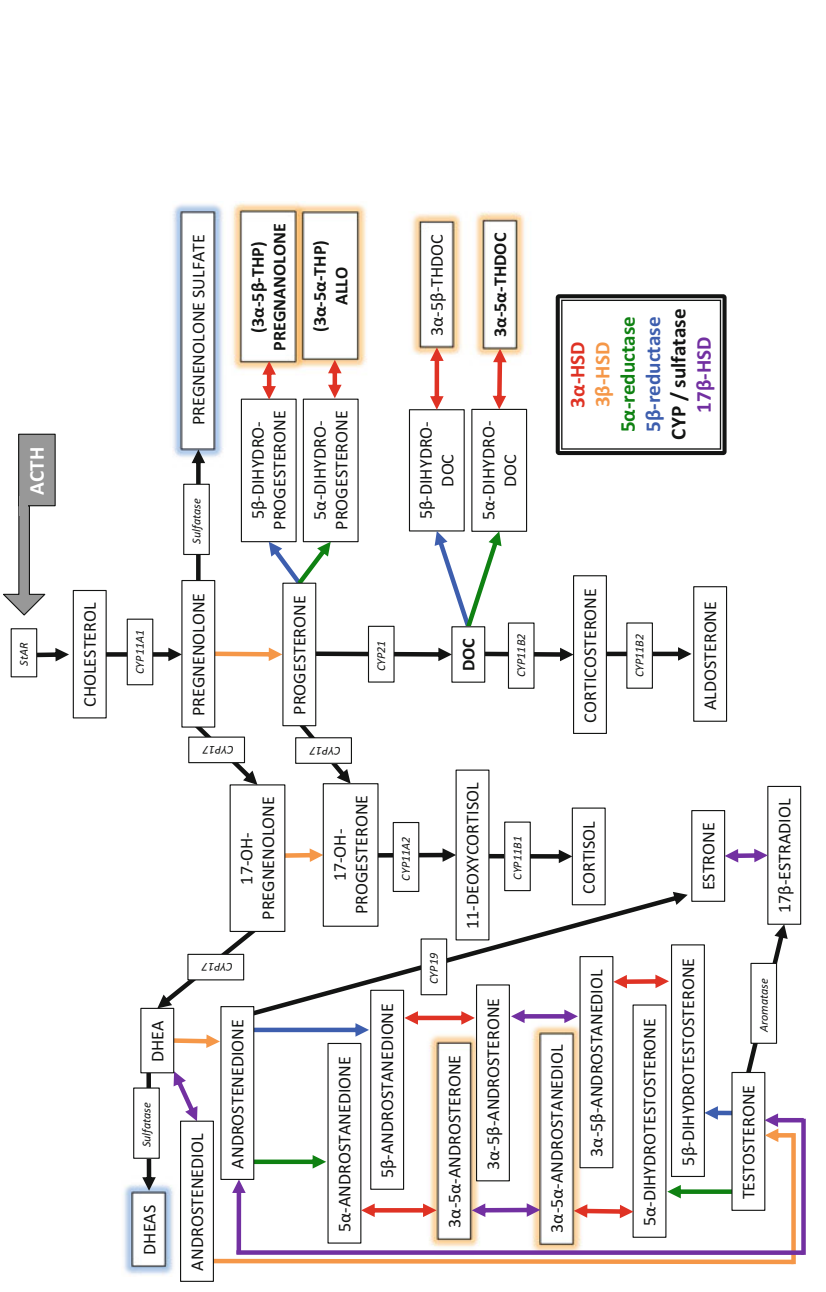

Fig. 1 Biosynthetic pathway of neurosteroids. Parent steroids and neurosteroids described in this chapter are depicted. Colored arrows refer to distinct enzymes, which are identified in the figure inset. Neurosteroids that are potent positive allosteric modulators of $GABA_A$ receptors are highlighted with orange, with the most potent shown in bold text. Neurosteroids that are negative modulators of $GABA_A$ receptors are highlighted with blue. This figure was modified from Mellon and Vaudry (2001), Porcu et al. (2009), and Snelling et al. (2014). *ACTH* adrenocorticotropic hormone, *ALLO* allopregnanolone, *CYP* cytochrome P450, *DHEA* dehydroepiandrosterone, *DOC* deoxycorticosterone, *HSD* hydroxysteroid dehydrogenase, *StAR* steroidogenic acute regulatory protein, *THDOC* tetrahydrodeoxycorticosterone, *THP* tetrahydroprogesterone

ability of neurosteroids to modulate alcohol's acute and chronic effects (e.g., Finn et al. 2004, 2010; Helms et al. 2012; Morrow et al. 2001, 2006, 2009; Porcu and Morrow 2014). This chapter highlights recent advances pertaining to the modulatory effects of neurosteroids that are potent positive allosteric modulators of $GABA_A$Rs. Background on neurosteroid pharmacology and biosynthetic enzymes is provided as it relates to experimental findings. Data are summarized on alcohol and neurosteroid interactions across neuroanatomical regions and models of alcohol intoxication, consumption, dependence, and withdrawal.

2 Neurosteroid Chemistry and Pharmacology

2.1 Actions on $GABA_A$ Receptors ($GABA_A$Rs)

The progesterone metabolites, ALLO and pregnanolone (3α,5β-THP), and the deoxycorticosterone (DOC) metabolite, 3α,5α-tetrahydrodeoxycorticosterone (5α-THDOC), are the three most potent positive modulators of $GABA_A$Rs characterized to date (Table 1), as they enhance $GABA_A$R-mediated inhibition with nanomolar potencies, directly activate $GABA_A$Rs with micromolar potencies, and interact with known modulatory sites on $GABA_A$Rs in a noncompetitive manner (Belelli and Lambert 2005; Belelli et al. 1990; Carver and Reddy 2013, 2016; Gee et al. 1988; Morrow et al. 1987; Paul and Purdy 1992; Purdy et al. 1990; Veleiro and Burton 2009). The testosterone metabolite 3α,5α-androstanediol and the dehydroepiandrosterone (DHEA) metabolite 3α,5α-androsterone potentiate

Table 1 Neurosteroids and actions on $GABA_A$ receptors

Neurosteroid	$GABA_A$ receptor action	Parent steroid
ALLO (allopregnanolone; 3α,5α-tetrahydroprogesterone; 3α,5α-THP)	Positive allosteric agonist	Progesterone
Pregnanolone (3α,5β-THP)	Positive allosteric agonist	Progesterone
5α-THDOC (3α,5α-tetrahydrodexoycorticosterone)	Positive allosteric agonist	DOC (deoxycorticosterone)
3α,5α-androstanediol	Positive allosteric agonist	Testosterone
3α,5α-androsterone	Positive allosteric agonist	DHEA (dehydroepiandrosterone)
PS (pregnenolone sulfate)	Noncompetitive antagonist	Pregnenolone
DHEAS (dehydroepiandrosterone sulfate)	Noncompetitive antagonist	DHEA

Neurosteroids that are positive allosteric agonists enhance $GABA_A$ receptor ($GABA_A$R)-mediated inhibition with nanomolar potencies, directly activate $GABA_A$Rs with micromolar potencies, and interact with known modulatory sites on $GABA_A$Rs in a noncompetitive manner. ALLO, pregnanolone, and 5α-THDOC are the three most potent positive modulators of $GABA_A$Rs characterized to date. The addition of a sulfate group at C-3 (e.g., to pregnenolone and DHEA) produces neurosteroids that antagonize $GABA_A$R-mediated inhibition in a noncompetitive manner, so these neurosteroids are noncompetitive antagonists

$GABA_A$Rs (Table 1), but with lower potency than ALLO and 5α-THDOC (Carver and Reddy 2013, 2016; Porcu et al. 2016). Notably, the interaction of these neurosteroids with $GABA_A$Rs is stereospecific, in that the two key features necessary for activity are a 5α- or 5β-reduced steroid A-ring and a 3α-hydroxyl group. The 3β-hydroxy analogues are devoid of activity or exhibit a partial inverse agonist profile. In addition to this structural specificity, elegant work by Hosie and colleagues determined that specific amino acid residues within the $GABA_A$R α subunits are critical for neurosteroid potentiation and that distinct residues within the α-β subunit interface are important for direct activation (Hosie et al. 2006, 2009), providing unequivocal confirmation of neurosteroid binding sites on $GABA_A$Rs. Importantly, the positive modulatory effect of neurosteroids at $GABA_A$Rs is relatively specific, in that these steroids do not interact with any other neurotransmitter receptor in the nanomolar to low micromolar concentration range. Interactions of the pregnane neurosteroids at ionotropic nicotinic acetylcholine, serotonin type 3, *N*-methyl-D-aspartate (NMDA), and metabotropic sigma 1 receptors occur within the 10–100 μM range (see Finn and Purdy 2007; Rupprecht and Holsboer 1999) and will not be discussed, because they are unlikely to have physiological relevance even under challenge conditions (i.e., stress or pregnancy; see Sect. 2.3).

Nanomolar concentrations of neurosteroids potentiate phasic and tonic forms of $GABA_A$R-mediated inhibition (e.g., Belelli and Lambert 2005; Carver and Reddy 2016; Helms et al. 2012; Herd et al. 2007; Zorumski et al. 2013), indicating that both synaptic and extrasynaptic $GABA_A$Rs possess sensitivity to neurosteroids and contribute to the overall ability of neurosteroids to modulate central nervous system (CNS) excitability. Moreover, physiologically relevant concentrations of ALLO affect presynaptic $GABA_A$Rs that are located on GABAergic or glutamatergic terminals to increase GABA (e.g., Park et al. 2011; also reviewed in Herd et al. 2007) or glutamate (e.g., Iwata et al. 2013) release, respectively, but it is not known whether all $GABA_A$R-active neurosteroids exert similar influences on presynaptic GABA and glutamate release. Thus, brain regional differences in the anatomical localization of presynaptic and postsynaptic $GABA_A$Rs could produce mixed effects of neurosteroids on CNS excitability.

Steroids with GABA-negative actions also have been reported [e.g., pregnenolone sulfate (PS) and DHEAS as the prototypical steroids with a sulfate at C-3], with the demonstration that PS and DHEAS antagonized GABA-gated chloride uptake and conductance in a noncompetitive manner (Table 1; discussed in detail in Finn and Purdy 2007). It is interesting that sulfated and unsulfated pregnane neurosteroids (e.g., ALLO) have opposing effects on $GABA_A$R function (Park-Chung et al. 1999). Thus, even though sulfation of steroids is a major enzymatic reaction to facilitate steroid excretion, it also can change the pharmacological activity of steroids (Mellon and Vaudry 2001). While the presence of sulfated steroids in the mammalian brain is still a matter of controversy (Do Rego et al. 2009; Finn and Purdy 2007), it is possible that the addition and removal of the sulfate group could be a critical control point for neurosteroid modulation of neurotransmitter receptors (see Gibbs and Farb 2000).

2.2 Neurosteroid Synthesis and Enzyme Distribution

Most of the enzymes present in the adrenals, gonads, and placenta have been found in the brain, and steroid synthesis is dependent on the tissue-, cell-, and developmentally specific expression of these enzymes (reviewed in Mellon and Vaudry 2001). As depicted in Fig. 1, the first rate-limiting step in steroid synthesis is the conversion of cholesterol to pregnenolone via the P450 side chain cleavage (P450scc or *CYP11A1*) enzyme upon the translocation of cholesterol from the outer to the inner mitochondrial membrane by the chaperone proteins steroidogenic acute regulatory protein (StAR; Stocco 2000) and translocator protein 18 kDa (TSPO; formerly peripheral or mitochondrial benzodiazepine receptor; Papadopoulos et al. 2006). And evidence confirms brain regional expression of StAR and P450scc (Kimoto et al. 2001; King et al. 2002). Then, through sequential steps, pregnenolone is converted to ALLO via 3β-hydroxysteroid dehydrogenase (HSD), 5α-reductase, and 3α-HSD, with progesterone and 5α-dihydroprogesterone as intermediates (Fig. 1). The reduction of progesterone, testosterone, and DOC via 5α-reductase is another rate-limiting step for neurosteroid production (Celotti et al. 1997). Within the CNS, 5α-reductase has been detected in neurons, astrocytes, and glia, and the predominant isoform is type 1 (see Mellon and Vaudry 2001). We found that 5α-reductase expression is widely distributed throughout mouse brain, with highest expression in specific regions of the cerebral cortex, hippocampus, thalamus, hypothalamus, and amygdala (Roselli et al. 2011). Agis-Balboa et al. (2006) demonstrated that 5α-reductase and 3α-HSD are co-localized in cortical, hippocampal, and olfactory bulb glutamatergic principal neurons and in some output neurons of the amygdala and thalamus as well as in principal GABAergic output neurons such as striatal medium spiny, reticular thalamic nucleus, and cerebellar Purkinje neurons, but not in cortical and hippocampal GABAergic interneurons. Thus, $GABA_AR$-active neurosteroids likely have important paracrine and autocrine effects on neuronal activity (discussed in Agis-Balboa et al. 2006).

2.3 Brain and Peripheral Sources

$GABA_AR$-active neurosteroids are synthesized from the metabolism of progesterone, DOC, testosterone, and DHEA (Fig. 1, Table 1), and a number of studies have established that the enzymes identified in classic steroidogenic tissues are also found in the nervous system (see Do Rego et al. 2009; Mellon and Vaudry 2001) and are maintained in the brain after gonadectomy (GDX) and adrenalectomy (ADX) in male and female rats (Kim et al. 2003). Thus, it is generally accepted that brain neurosteroid levels reflect a combination of neuroactive compounds produced de novo as well as peripherally derived precursor steroids that are metabolized to neurosteroids in the brain. For this reason, it has been proposed that the definition of the term "neurosteroid" be broadened to include both sources of neuroactive steroids (Mellon and Vaudry 2001). So, we will use the term "neurosteroid" throughout this chapter.

Concentrations of the progesterone derivative ALLO, the most potent positive allosteric modulator of $GABA_A$Rs (e.g., Belelli and Lambert 2005), are detected in the brain or plasma/serum of the rat, mouse, dog, monkey, and human (e.g., see Finn et al. 2004; Porcu and Morrow 2014 and references therein; also Caruso et al. 2013; Cozzoli et al. 2014; Hill et al. 2005; Jensen et al. 2017; Porcu et al. 2009, 2010; Romeo et al. 1996; Snelling et al. 2014). In addition, brain ALLO level is detectable in ADX animals and is higher than plasma level in intact animals (see Finn and Purdy 2007).

Evidence indicates that endogenous $GABA_A$R-active neurosteroids such as ALLO and 5α-THDOC can reach levels that are within the range of concentrations previously shown to potentiate the in vitro action of GABA at $GABA_A$Rs. ALLO and 5α-THDOC levels fluctuate in response to acute stress in rodents, with significant increases following ambient temperature swim, foot shock, or CO_2 inhalation in male rats to the equivalent of 10–30 nM (Barbaccia et al. 2001; Purdy et al. 1991; Reddy and Rogawski 2002) and significant increases following restraint, tail suspension, or predator odor exposure in male and female mice to the equivalent of 10–20 nM (Cozzoli et al. 2014). Notably, the swim stress-induced increase in 5α-THDOC exerted an anticonvulsant effect (Reddy and Rogawski 2002). Plasma ALLO levels also were increased significantly during PhD examination stress in males and females (Droogleever Fortuyn et al. 2004). In the female rodent, brain and plasma levels of ALLO temporally follow those of progesterone, with levels in the range of 10–30 nM during estrus and increasing to 100 nM during pregnancy (e.g., Concas et al. 1998; Finn and Gee 1994; Paul and Purdy 1992). Taken in conjunction with the finding that manipulation of local ALLO levels within the hippocampus and thalamus significantly altered $GABA_A$R-mediated inhibition (Belelli and Herd 2003; Brown et al. 2015), the results suggest that fluctuations in endogenous neurosteroid levels are physiologically relevant (e.g., Belelli and Lambert 2005).

3 Physiological Significance of Neurosteroid Fluctuations and Interaction with Alcohol

Neurosteroids that are positive modulators of $GABA_A$Rs possess anesthetic, hypnotic, anticonvulsant, anxiolytic, antidepressant, analgesic, and amnesic effects (see reviews by Finn and Purdy 2007; Gasior et al. 1999; Porcu et al. 2016). These behavioral responses are consistent with in vitro evidence and suggest that GABAergic steroids modify the functioning of central $GABA_A$Rs in vivo. Thus, if the findings with exogenous administration of $GABA_A$R-active neurosteroids are indicative of $GABA_A$R sensitivity to endogenous concentration, then endogenous neurosteroids may participate in the physiological control of CNS excitability. Consistent with this idea, use of a 5α-reductase inhibitor to decrease endogenous ALLO levels was proconvulsant (Gililland-Kaufman et al. 2008) and blocked the anticonvulsant effect produced by a stress-induced increase in 5α-THDOC levels (Reddy and Rogawski 2002).

Based on the similar pharmacological profile of $GABA_AR$-active neurosteroids and alcohol, there is interest in the ability of neurosteroid fluctuations to influence alcohol sensitivity. Acute administration of alcohol (1–2.5 g/kg) produces a steroidogenic effect, measured by a significant increase in levels of ALLO and 5α-THDOC and their precursors in the brain and plasma of rodents and in plasma ALLO levels in humans, although some conflicting results in mice and humans have been reported (see reviews by Finn et al. 2004; Kumar et al. 2009; Morrow et al. 2006; Porcu and Morrow 2014) and increases have not been detected in monkeys (reviewed in Helms et al. 2012). Notably, these alcohol-induced elevations in $GABA_AR$-active neurosteroids reach concentrations that enhance $GABA_AR$ inhibition and influence several behavioral effects of alcohol (Finn et al. 2004; Kumar et al. 2009; Morrow et al. 2006; Porcu and Morrow 2014), indicating that the alcohol-induced increases in $GABA_AR$-active neurosteroid levels are physiologically relevant. For example, a reduction in $GABA_AR$-active neurosteroid levels via pretreatment with a 5α-reductase inhibitor or prior ADX significantly reduced alcohol's anticonvulsant, sedative, amnesic, anxiolytic, antidepressant-like, and pro-aggressive effects. Moreover, alcohol's steroidogenic effect in the rat was demonstrated in hippocampal slices in vitro (Sanna et al. 2004), was enhanced in animals with a chronic stress-induced decrease in endogenous ALLO levels (Serra et al. 2003), and was associated with increased StAR expression in the cortex, hypothalamus, and hippocampus (Kim et al. 2003; Serra et al. 2006). Subsequent studies identified two independent mechanisms contributing to alcohol's steroidogenic effect in the rat: pituitary activation to release adrenocorticotropic hormone (ACTH) and de novo adrenal StAR formation (Boyd et al. 2010a). Collectively, these data indicate that fluctuations in $GABA_AR$-active neurosteroids influence sensitivity to many behavioral effects of alcohol.

Neurosteroids also possess rewarding properties in rodents and monkeys. Rodents exhibit conditioned place preference to ALLO, display preference for ALLO solutions over water, and consume anxiolytic doses of ALLO (Finn et al. 1997, 2003; Sinnott et al. 2002). And one study in monkeys determined that pregnanolone functioned as a reinforcer in animals trained to administer this neurosteroid intravenously (Rowlett et al. 1999). However, in contrast to the ability of neurosteroids to contribute to several behavioral effects of alcohol, as described above, ALLO levels did not influence alcohol-induced conditioned place preference in mice (Gabriel et al. 2004; Murphy et al. 2006).

Drug discrimination procedures indicate that neurosteroids that are positive modulators of $GABA_ARs$ have alcohol-like discriminative stimulus properties in rodents and nonhuman primates, whereas neurosteroids that are negative modulators of $GABA_ARs$ do not substitute for alcohol (reviewed in Morrow et al. 2006). In female macaques, lower doses of ALLO substituted for alcohol during the luteal versus follicular phase of the menstrual cycle (Grant et al. 1997), suggesting that females have enhanced sensitivity to alcohol's subjective effects when progesterone and ALLO levels are high. In male rodents, ALLO promoted reinstatement of extinguished alcohol self-administration (Finn et al. 2008; Nie and Janak 2003), and similar results were found with ganaxolone (GAN; Ramaker et al. 2014), the 3β-methylated analogue of ALLO that has a similar pharmacological profile as

ALLO but a half-life about three to four times longer when given systemically (Carter et al. 1997). And consistent with the importance of the GABAergic system in regulating alcohol consumption, systemic administration of ALLO and GAN produced dose-dependent and biphasic changes in alcohol intake in a variety of procedures in male rodents (see Morrow et al. 2006; Ramaker et al. 2015), with low doses enhancing, and higher doses reducing, alcohol self-administration. The 5α-reductase inhibitor finasteride (FIN) also suppressed alcohol consumption in male mice via different effects on the microarchitecture of alcohol drinking than ALLO, and female mice exhibited a lower sensitivity to the modulatory effects of ALLO and FIN on alcohol drinking (reviewed in Finn et al. 2010). Interestingly, FIN reduced the subjective effects of alcohol in humans, an effect that was dependent on $GABA_AR$ α2 subunit genotype (Pierucci-Lagha et al. 2005), and the 5α-reductase inhibitor dutasteride reduced alcohol consumption in male subjects classified as heavy drinkers (Covault et al. 2014). Collectively, there is a strong relationship between neurosteroid levels and alcohol consumption, subjective effects, and measures of relapse, but additional studies are necessary to better understand the genetic factors and mechanisms underlying sex differences in different species.

Following the induction of physical dependence in rodents, differences in sensitivity to the anticonvulsant effect of $GABA_AR$-active neurosteroids or synthetic neurosteroids have been identified (see Finn et al. 2010 and references therein; also Cagetti et al. 2004; Devaud et al. 1995). Specifically, rodents with a low withdrawal convulsive profile (e.g., rats, C57BL/6J mice, Withdrawal Seizure-Resistant (WSR) selected line) exhibit increased sensitivity to the anticonvulsant effect of ALLO and alphaxalone versus controls. In contrast, mice with a high withdrawal convulsive profile (e.g., DBA/2J mice, Withdrawal Seizure-Prone (WSP) selected line) exhibited tolerance to ALLO's anticonvulsant effect during withdrawal when compared to sensitivity in controls. Notably, these changes in sensitivity corresponded to leftward (rats) and rightward (WSP mice) shifts in functional sensitivity of $GABA_ARs$ to ALLO, and similar behavioral results were found in males and females. These findings suggest that the plasticity of $GABA_ARs$ during alcohol withdrawal may differ between alcohol withdrawal seizure-prone and withdrawal seizure-resistant genotypes, particularly with regard to ALLO sensitivity.

4 Alcohol and Neurosteroid Interactions Across Neuroanatomical Regions

As described above, acute stress (Sect. 2.3) and acute alcohol administration (Sect. 3) can significantly increase $GABA_AR$-active neurosteroid levels and influence behavior and alcohol sensitivity. In contrast, chronic stress (e.g., social isolation) produces a consistent decrease in endogenous ALLO levels that is associated with an increase in anxiety-related behavior and contextual fear responses, decreased sensitivity to the hypnotic effects of $GABA_AR$-active

compounds, and an enhanced steroidogenic effect to acute alcohol administration and acute stress exposure (see Biggio et al. 2014; Finn and Purdy 2007; Pibri et al. 2008; Serra et al. 2003). Thus, the period of exposure to stress may produce opposite effects on endogenous neurosteroid levels and subsequent physiological responses.

Endogenous neurosteroid levels influence the rebound neuronal hyperexcitability seen during withdrawal from a hypnotic alcohol dose (i.e., acute withdrawal response). Specifically, ADX/GDX to decrease endogenous neurosteroid levels increased acute withdrawal-induced convulsive behavior, which was reversed by replacement with $GABA_AR$-active steroid precursors and metabolism to GABAergic neurosteroids (Kaufman et al. 2010). Additionally, chronic alcohol exposure and withdrawal is associated with a decrease in $GABA_AR$ inhibition mediated by a variety of factors that includes a reduction in the steroidogenic effect of acute alcohol administration (Boyd et al. 2010b), functional changes in $GABA_AR$ properties, and a decrease in endogenous ALLO levels (see Finn et al. 2004; Kumar et al. 2009). In rodents, monkeys, and humans, withdrawal decreases ALLO levels in plasma and several brain regions (Beattie et al. 2017; Cagetti et al. 2004; Hill et al. 2005; Jensen et al. 2017; Maldonado-Devincci et al. 2014; Romeo et al. 1996; Snelling et al. 2014). For example, a withdrawal-induced decrease in hippocampal ALLO levels was associated with a significant increase in anxiety and impairment in hippocampal-dependent memory function in male rats (Cagetti et al. 2003, 2004). In small cohorts of male and female alcoholics, the decrease in ALLO and 5α-THDOC levels corresponded to an increase in the subjective ratings of anxiety and depression during days 4–5 of withdrawal, versus controls (Hill et al. 2005; Romeo et al. 1996). Furthermore, an examination of the pattern of changes in several $GABA_AR$-active neurosteroid levels in mouse brain and plasma during withdrawal revealed a broad and complex dysregulation in neurosteroid biosynthesis (Jensen et al. 2017; Snelling et al. 2014). The brain versus plasma differences in the withdrawal-induced changes are consistent with the findings that basal neurosteroid levels in plasma do not simply reflect levels in cortex and hippocampus (Caruso et al. 2013) and argue for independent regulation of neurosteroid synthesis in the periphery and brain during withdrawal. Thus, chronic alcohol withdrawal produces a consistent reduction in endogenous ALLO levels and dysregulation in neurosteroid synthesis that may be associated with increased cellular excitability and increased aversive behavioral effects (e.g., anxiety, depression, convulsive activity).

4.1 Hypothalamic-Pituitary-Adrenal (HPA) Axis Stress Circuit

Acute stress stimulates the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (PVN), ACTH from the pituitary, and glucocorticoids from the adrenal (cortisol in primates and corticosterone in rodents). And as mentioned in Sect. 2.3, acute stressors also increase levels of the $GABA_AR$-active neurosteroids ALLO and 5α-THDOC (see Finn and Purdy 2007).

Administration of ALLO reduced anxiety that was induced by CRH (Patchev et al. 1994) in addition to exerting actions within the hypothalamus to dampen the activity of the HPA axis (Patchev et al. 1994, 1996). These data demonstrate that GABAergic neurosteroids participate in the activity of the HPA axis.

Acute alcohol stimulates the HPA axis (e.g., Lee et al. 2004; Ogilvie et al. 1997; Rivier and Lee 1996) and synthesis of neurosteroids (see Sect. 3). With repeated alcohol exposure, both the HPA axis and neurosteroid synthesis show tolerance (Boyd et al. 2010b; Richardson et al. 2008). In cynomolgus macaques, disinhibition of the PVN (following naloxone, a μ-opioid receptor antagonist), but not stimulation of the pituitary (ovine-CRH) or adrenal gland (exogenous ACTH), increased pregnenolone levels (see Fig. 1, Porcu et al. 2006), indicating the PVN plays a role in regulation of neurosteroid synthesis. Interestingly, DOC secretion was increased following pituitary stimulation (ovine-CRH), but not disinhibition of the PVN (naloxone; Jimenez et al. 2017), suggesting possible differential regulation of neurosteroid precursors by activation of the HPA axis. Regulation of DOC by the HPA axis was altered during the induction of alcohol drinking using schedule-induced polydipsia, where the response to ovine-CRH was blunted and the response to naloxone was potentiated (Jimenez et al. 2017), hinting that an additive effect of schedule-induced stress and alcohol consumption may influence the relationship between the HPA axis and neurosteroid synthesis. And, de novo synthesis has been demonstrated following alcohol exposure in the PVN (Fig. 2). Acute alcohol administration (2 g/kg) significantly increased ALLO immunohistochemistry (IHC) in rats, and this effect was independent of the adrenal glands (Cook et al. 2014a, b).

Although alcohol interacts with several components of the HPA axis, the increase in HPA axis activity relies on activation of the PVN (Lee et al. 2004). The majority of synaptic connections within the PVN are GABAergic and glutamatergic (Miklós and Kovács 2002; van den Pol et al. 1990). Tonic inhibition of the PVN likely occurs via glutamatergic forebrain afferents that increase GABA release in the PVN or activation of the PVN via inhibition by upstream GABAergic projection neurons (Fig. 2, Cullinan et al. 2008). Thus, $GABA_AR$-active neurosteroids are particularly well suited to modulate activity in the PVN, since physiological concentrations of ALLO (10–100 nM) inhibit the output of PVN neurons (i.e., CRH release) via a potentiation of $GABA_ARs$ (Gunn et al. 2011). This ability of $GABA_AR$-active neurosteroids to inhibit CRH release could contribute to a termination of the stress response.

4.2 Extra-hypothalamic Stress Circuit

Amygdala: The amygdala contributes to fear- and anxiety-like behavior as well as HPA axis activity. Its role in alcohol use disorders (AUDs) is rapidly gaining attention (see Gilpin et al. 2015). The amygdala plays a pivotal role in the assessment of and response to danger, with connections to the cortex and locus coeruleus and projections to the striatum, hypothalamus, midbrain, and brainstem (simplified circuit in Fig. 2; see Gilpin et al. 2015 for details on amygdala microcircuitry and

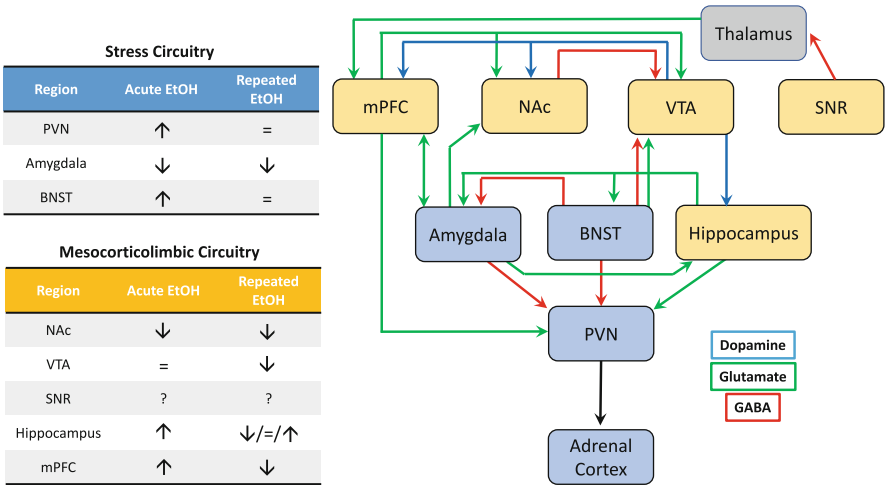

Fig. 2 Simplified stress (blue) and mesocorticolimbic (yellow) circuitry and summary of the effects of acute and repeated alcohol administration and withdrawal. Glutamatergic, GABAergic, and dopaminergic projections are indicated by green, red, and blue arrows, respectively, in this simplified representation of the neuroanatomical regions described in the chapter. The black arrow from the paraventricular nucleus of the hypothalamus (PVN) to the adrenal cortex reflects a streamlined depiction of the hypothalamic-pituitary-adrenal axis. The tables in the figure summarize the overall effect(s) of acute and repeated alcohol administration and withdrawal on allopregnanolone (ALLO) levels, with increases (↑), decreases (↓), no change (=), or unknown (?) shown. Mixed results of chronic alcohol administration and withdrawal on hippocampal ALLO levels have been reported. An alcohol-induced increase in ALLO levels would enhance $GABA_A$ receptor-mediated inhibition, whereas a decrease in ALLO levels would produce the opposite effect. *BNST* bed nucleus of the stria terminalis, *NAc* nucleus accumbens, *mPFC* medial prefrontal cortex, *PVN* paraventricular nucleus of the hypothalamus, *SNR* substantia nigra reticulata, *VTA* ventral tegmental area

projection neurons). Microinfusion of ALLO into the central nucleus of the amygdala (CeA) decreased anxiety-like behavior in rodents (Akwa et al. 1999; Engin and Treit 2007). Electrophysiologically, ALLO's effect on evoked $GABA_AR$-mediated currents appeared to depend on network activity and involved NMDA-mediated currents (Wang et al. 2007). Recent imaging results indicate that administration of pregnenolone, which increased ALLO levels, was associated with reduced activity in the amygdala, increased activity in the dorsal medial prefrontal cortex (mPFC), enhanced connectivity between the two regions, and less self-reported anxiety (Sripada et al. 2013). These data suggest that ALLO modulates emotion neurocircuits.

In response to an acute alcohol injection (2 g/kg), ALLO IHC within the CeA was significantly decreased in rats, independent of ADX (Cook et al. 2014a, b). Similarly, mice exposed to chronic intermittent alcohol (CIE) had reduced ALLO in the CeA at 8-h but not 72-h withdrawal when compared to controls (Maldonado-Devincci et al. 2014). In male cynomolgus monkeys that had been consuming alcohol daily for over 12 months, there was a significant decrease in plasma ALLO levels and in ALLO IHC in the lateral and basolateral amygdala versus

controls (Beattie et al. 2017). A significant negative correlation between ALLO IHC in the lateral and basolateral amygdala and average daily ethanol consumption suggested that long-term high alcohol consumption dampens ALLO IHC. Collectively, alcohol exposure reveals a consistent reduction in endogenous ALLO levels in the amygdala (Fig. 2) that may be associated with increased cellular excitability and high prior alcohol consumption or exposure.

Bed nucleus of the stria terminalis (BNST): The BNST is important for fear- and anxiety-like behavior, serves as a relay from the amygdala, cortex and hippocampus to the PVN (Lebow and Chen 2016), and has a well-established role in AUDs (Kash 2012). Both the BNST and CeA have a high density of $GABA_A$Rs, and GABA is the predominant co-transmitter in CRH^+ neurons in these brain regions (Partridge et al. 2016). ALLO IHC increased in the BNST of rats following acute alcohol administration (Fig. 2), and this effect was independent of adrenal sources (Cook et al. 2014a, b). In contrast, withdrawal from CIE exposure did not alter ALLO IHC in the BNST in mice (Fig. 2, Maldonado-Devincci et al. 2014). As noted above, the response to acute alcohol and to CIE both resulted in a decrease in ALLO IHC within the CeA, suggesting that the effect of acute and chronic alcohol exposure may be regionally specific.

4.3 Mesocorticolimbic Circuit

As recently reviewed (Koob and Volkow 2010), drug addiction can be comprised of binge/intoxication, withdrawal/negative affect, and craving stages that recruit different neuroanatomical regions within the mesocorticolimbic circuit. An excellent review of synaptic and extrasynaptic $GABA_A$R isoforms important in the mesocorticolimbic reward circuitry also is available (Stephens et al. 2017). States of reward and aversion are encoded by the activity of GABAergic medium spiny neurons (MSNs) in the nucleus accumbens (NAc; see Stephens et al. 2017), which receives glutamatergic inputs from hippocampus, amygdala, and cortical areas. The receipt of important limbic information from the amygdala, frontal cortex, and hippocampus is integrated in the NAc and converted to motivational action through outputs via the direct striatonigral and the indirect striatopallidal pathways (see Koob and Volkow 2010; Stephens et al. 2017). The location of both synaptic and extrasynaptic $GABA_A$R isoforms throughout this circuitry suggests the possibility of spatially controlled regulation of $GABA_A$R function by neurosteroids and alcohol in motivational and withdrawal effects.

Nucleus accumbens (NAc): Approximately 97% of NAc neurons (principal neurons, MSNs, and interneurons) utilize the neurotransmitter GABA, and infusion of $GABA_A$R agonists or antagonists into the NAc shell (e.g., Eiler and June 2007; Hyytiä and Koob 1995; Stratford and Wirtshafter 2011) as well as viral knockdown of $GABA_A$R δ or α4 subunits (Nie et al. 2011; Rewal et al. 2009, 2012) significantly decreased alcohol intake in rodents. Intra-NAc shell administration of the synthetic neurosteroid GAN also significantly decreased alcohol intake, an effect that was similar to that observed following intracerebroventricular administration of GAN or

ALLO (Ford et al. 2007; Ramaker et al. 2015). Taken in conjunction with the finding that intra-NAc ALLO substituted for the discriminative stimulus effects of systemic alcohol (Hodge et al. 2001), these results provide evidence for the sufficiency of $GABA_A$Rs in the NAc to influence alcohol's subjective, motivational, and consummatory effects (see also Sect. 3).

Acute administration of alcohol reduced ALLO IHC in the NAc core-shell border (Fig. 2), an effect that was independent of peripheral sources (Cook et al. 2014a, b). Withdrawal (72-h) from CIE also decreased ALLO IHC in the NAc core (Fig. 2; Maldonado-Devincci et al. 2014), suggesting that subregion differences may exist in the regulation of neurosteroid synthesis.

Ventral tegmental area (VTA): Microinjection of a viral vector to overexpress P450scc significantly increased ALLO IHC in the VTA and decreased alcohol self-administration (Cook et al. 2014c). ALLO was localized in neurons, primarily in all tyrosine hydroxylase positive neurons, which could reduce activity in cells that project to the NAc, mPFC, or lateral habenula (Cook et al. 2014c). Acute alcohol injection did not alter ALLO levels (Cook et al. 2014b), but CIE produced a persistent reduction in ALLO IHC in the VTA (Fig. 2) at 8-h and 72-h withdrawal (Maldonado-Devincci et al. 2014). Microinjection of ALLO produced an anticonvulsant effect in naïve mice that was reduced during alcohol withdrawal in mice with a high withdrawal phenotype, and microinjection of FIN (5α-reductase inhibitor) during the induction of physical dependence (to determine the effect of a decrease in local ALLO levels on the expression of withdrawal) enhanced alcohol withdrawal severity (Tanchuck et al. 2013). Microinjections in the withdrawal studies were localized to the posterior VTA, which also projects to the SN compacta and connects the striatum to the output nuclei of the basal ganglia via the indirect pathway (discussed in Tanchuck et al. 2013). Collectively, the results suggest that manipulation of $GABA_A$R-active neurosteroid levels in the VTA influences alcohol self-administration and convulsive activity during withdrawal.

Substantia nigra reticulata (SNR): The SNR is important in the propagation of convulsive activity, as it is one of the two major output nuclei of the basal ganglia, with GABAergic projections to superior colliculus, brainstem nuclei, and thalamus (see Tanchuck et al. 2013). ALLO infusion into SNR exerted a potent anticonvulsant effect in naïve mice, at lower doses than observed following microinjection into the VTA (Tanchuck et al. 2013). It is possible that the greater sensitivity to ALLO's anticonvulsant effect in the SNR than in the VTA reflects direct versus indirect effects, respectively, on $GABA_A$R-mediated output of the basal ganglia. Similar to what was observed in the VTA, there was a diminished anticonvulsant effect during alcohol withdrawal in mice with a high withdrawal phenotype (Tanchuck et al. 2013). Microinjection of FIN during the induction of physical dependence did not influence alcohol withdrawal severity but produced a delayed proconvulsant effect in naïve mice (Tanchuck et al. 2013). Overall, these results provide support for the sufficiency of the SNR in mediating the anticonvulsant effect of ALLO in naïve mice and the behavioral tolerance to ALLO's anticonvulsant effect during withdrawal in mice with a high withdrawal phenotype.

Hippocampus: Acute alcohol administration increased ALLO IHC in the hippocampal CA1 pyramidal cell layer and dentate gyrus (DG) polymorphic layer (Fig. 2), which was independent of the adrenals (Cook et al. 2014a, b). In contrast, withdrawal from repeated CIE did not alter ALLO labeling in either of these subregions, but there was an increase in ALLO IHC in the CA3 pyramidal layer (Maldonado-Devincci et al. 2014). Studies with dissected hippocampal tissue reported decreased ALLO levels during withdrawal in rats (Fig. 2; Cagetti et al. 2004) and divergent changes in several $GABA_AR$-active neurosteroid levels during withdrawal in mice that were unrelated to a convulsive phenotype (Jensen et al. 2017). However, microinjection of ALLO into CA1 produced a potent anticonvulsant effect in WSP mice (Gililland-Kaufman et al. 2008), with brain regional differences in sensitivity to the anticonvulsant effect in alcohol naïve mice (CA1 $\geq$ SNR $>$ VTA). In contrast, infusion of FIN into CA1 was proconvulsant (Gililland-Kaufman et al. 2008). These behavioral findings demonstrate that bi-directional manipulation of hippocampal ALLO levels produces opposite behavioral consequences that are consistent with alterations in $GABA_AR$ inhibitory tone in naïve mice. During withdrawal, WSP mice were tolerant to the anticonvulsant effect of intra-CA1 ALLO, consistent with results following systemic injection (Finn et al. 2006), and intra-CA1 FIN during the development of physical dependence significantly increased alcohol withdrawal severity (Gililland-Kaufman et al. 2008). Thus, alcohol withdrawal rendered WSP mice less sensitive to ALLO's anticonvulsant effect and more sensitive to FIN's proconvulsant effect, suggesting an alteration in the sensitivity of hippocampal $GABA_ARs$ in response to fluctuations in $GABA_AR$-active neurosteroids during withdrawal. Collectively, the microinjection results provide support for the sufficiency of the CA1 in mediating the anticonvulsant effect of ALLO in naïve mice and the behavioral tolerance to ALLO's anticonvulsant effect during withdrawal in WSP mice.

Medial prefrontal cortex (mPFC): The ability of an acute alcohol injection to increase ALLO levels in dissected mPFC of male rats has been well-documented (Fig. 2; see reviews by Morrow et al. 2006; Porcu and Morrow 2014). More recent work confirmed an alcohol-induced elevation in ALLO IHC in mPFC (Cook et al. 2014a, b) but determined that the increase was dependent on the adrenal glands (Cook et al. 2014a). This result differs from the independent regulation of neurosteroid synthesis after acute alcohol in other brain regions. In contrast, there was a sustained decrease in ALLO IHC in mPFC at 8-h and 72-h of withdrawal after CIE exposure (Fig. 2, Maldonado-Devincci et al. 2014). Measurement of several $GABA_AR$-active neurosteroid levels in dissected tissue determined that cortical levels of ALLO and other $GABA_AR$-active neurosteroids were decreased at 8-h withdrawal only in mice with a low withdrawal convulsive phenotype (Jensen et al. 2017). Levels of cortical $GABA_AR$-active neurosteroids were unchanged or increased in mice with a high withdrawal convulsive phenotype, which contrasts with the suppression in plasma ALLO levels during withdrawal in these genotypes (Jensen et al. 2017; Snelling et al. 2014) and argues for independent adrenal versus brain regional regulation of neurosteroid synthesis.

5 Conclusions

$GABA_AR$-active neurosteroids exert behavioral and physiological responses that are consistent with their ability to enhance $GABA_AR$-mediated inhibition. Microinjection and electrophysiological studies provide evidence for brain regional differences in sensitivity, which may reflect differences in $GABA_AR$ subunit composition or local synthesis and metabolism. Elegant studies indicate that locally produced neurosteroids in thalamocortical neurons enhanced $GABA_AR$-mediated inhibition (Brown et al. 2015) and that differences in neurosteroid metabolism in hippocampal DG versus CA1 produced predicted effects on GABAergic transmission (i.e., increased $GABA_AR$-mediated inhibition with increased neurosteroid level; Belelli and Herd 2003). Consistent with the idea that brain regional differences in neurosteroid synthesis and metabolism can influence effects of alcohol, the use of a viral vector to overexpress P450scc in the VTA, but not in the NAc, significantly increased ALLO levels and decreased alcohol self-administration (Cook et al. 2014c). Moreover, physiological concentrations of ALLO inhibit the output of PVN neurons via a potentiation of $GABA_ARs$ (Gunn et al. 2011), representing another mechanism to terminate the stress response via an inhibition of CRH release. Thus, local brain regional mechanisms to fine tune neuronal excitability exist, and additional studies will be important to further understand the physiological significance of these brain regional differences (see Fig. 2 for simplified circuitry). Then, strategies based on pharmacological agents or gene therapy tools that can increase neurosteroid levels directly in discrete brain regions may represent a promising area of research.

Acute and chronic alcohol administration and withdrawal produced species and brain regional differences in neurosteroid levels (Fig. 2), with many brain regional effects being independent of the adrenals, providing evidence for independent regulation between periphery and brain as well as across brain regions. The inverse relationship between levels of some neurosteroid enzymes and GABA (Do Rego et al. 2009) also may contribute to brain regional differences in regulation of neurosteroid synthesis as well as to sensitivity of $GABA_ARs$ to neurosteroids, since potentiation of $GABA_ARs$ with nanomolar concentrations of neurosteroids requires GABA. Additionally, the ability of alcohol to increase spontaneous and evoked GABA release in brain regions such as cerebellum, VTA, SN, and amygdala but not in the cortex, lateral septum, and thalamus (reviewed in Kelm et al. 2011) may indirectly influence brain regional differences in neurosteroid levels and $GABA_AR$ sensitivity to neurosteroids. Finally, the use of GAN, which has a similar pharmacological profile to ALLO but is resistant to oxidation at C-3, can help to distinguish whether reduced behavioral sensitivity to ALLO is due to enhanced metabolism or altered sensitivity of $GABA_ARs$ to neurosteroids. For instance, WSP mice exhibit tolerance to the anticonvulsant effect of ALLO and GAN during alcohol withdrawal, consistent with a decrease in functional sensitivity of $GABA_ARs$ during withdrawal (Finn et al. 2006; Nipper et al. 2017). In contrast, DBA/2J mice exhibited sensitivity to the anticonvulsant effect of GAN but not ALLO during alcohol withdrawal (Finn et al. 2000; Nipper et al. 2017), suggesting

that a withdrawal-induced change in ALLO metabolism may play a larger role than decreased sensitivity of $GABA_A$Rs to neurosteroids per se. As another example, sex differences in C57BL/6J mice in the ability of ALLO to decrease alcohol consumption may be due in part to enhanced ALLO metabolism in female mice, given that GAN was equally effective at decreasing alcohol intake in both male and female mice and that ALLO exerted a decrease in alcohol intake in female mice when the oxidation at C-3 was blocked (DA Finn and MM Ford, unpublished). Collectively, additional studies are necessary to further understand the interaction between alcohol's acute and chronic effects on neurosteroid levels and $GABA_A$R sensitivity to provide insight on whether pharmacological strategies targeting neurosteroid synthesis or using synthetic neurosteroid compounds that are resistant to metabolism (i.e., GAN) will be effective treatment approaches for AUD.

Administration of ALLO and GAN doses produces a fairly consistent suppression in alcohol consumption and self-administration (see Sect. 3), and in conjunction with the pharmacological properties of $GABA_A$R-active neurosteroids (e.g., anxiolytic, anticonvulsant, antidepressant; see Sect. 3), it has been hypothesized that elevations in neurosteroid levels may protect against the risk for alcohol dependence (see Morrow et al. 2006). Alcohol dependence and withdrawal are associated with a decrease in $GABA_A$R inhibition that is mediated by many factors, one of which is a fairly consistent reduction in endogenous ALLO levels (Fig. 2) that is accompanied by increased anxiety in rodents and increased ratings of anxiety and depression in humans (see Sect. 4). Given that altered neurosteroid synthesis or neurosteroid levels have been reported in patients with several mood disorders (see Finn and Purdy 2007; Porcu et al. 2016; Zorumski et al. 2013), it is possible that patients with comorbid AUD and mood disorders also exhibit a dysregulation in neurosteroid synthesis and that this suppression in neurosteroid levels contributes to the withdrawal/negative affect stage of addiction. One strategy to reduce relapse risk would be to offset the potential negative affective state with a synthetic neurosteroid such as GAN, which is in clinical trials for treatment of various forms of depression and epilepsy (clinicaltrial.gov). Genetic diversity in enzyme levels also should be considered, given the finding that individuals with the minor C-allele of the *SRD5A1* gene, which encodes the enzyme 5α-reductase-1, expressed both a higher ratio of dihydrotestosterone to testosterone and a decreased risk for alcohol dependence (Milivojevic et al. 2011), suggesting that a heightened level of $GABA_A$R-active neurosteroid production may be protective against the development of dependence. Certainly, alleles that decrease enzyme function and the biosynthesis of $GABA_A$R-active neurosteroids could exacerbate the risk of dependence. Thus, genetic differences in neurosteroid enzyme levels and biosynthesis are an important consideration for future studies examining the therapeutic potential of targeting neurosteroid biosynthesis.

Collectively, neurosteroids are extremely potent positive modulators of $GABA_A$Rs that can exhibit exquisite neuroanatomical control of $GABA_A$R-mediated inhibition and physiological and behavioral responses. Additional research is necessary to better understand the physiological significance of the complex interactions with alcohol's acute and chronic effects across brain regions and in the periphery. Future studies also

should determine the therapeutic potential of strategies to enhance neurosteroid synthesis or to administer synthetic neurosteroids for the treatment of AUD.

Acknowledgments Supported by NIH RO1 AA012439 and grants and resources from the Department of Veterans Affairs (DAF). We thank Dr. Matthew Ford for the critical commentary on the chapter.

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GABA and Glutamate Synaptic Coadaptations to Chronic Ethanol in the Striatum

Verginia C. Cuzon Carlson

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Abstract

Alcohol (ethanol) is a widely used and abused drug with approximately 90% of adults over the age of 18 consuming alcohol at some point in their lifetime. Alcohol exerts its actions through multiple neurotransmitter systems within the

V. C. Cuzon Carlson (✉)
Oregon National Primate Research Center, Beaverton, OR, USA
e-mail: cuzoncar@ohsu.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_98

brain, most notably the GABAergic and glutamatergic systems. Alcohol's actions on GABAergic and glutamatergic neurotransmission have been suggested to underlie the acute behavioral effects of ethanol. The striatum is the primary input nucleus of the basal ganglia that plays a role in motor and reward systems. The effect of ethanol on GABAergic and glutamatergic neurotransmission within striatal circuitry has been thought to underlie ethanol taking, seeking, withdrawal and relapse. This chapter reviews the effects of ethanol on GABAergic and glutamatergic transmission, highlighting the dynamic changes in striatal circuitry from acute to chronic exposure and withdrawal.

Keywords
Action control · Addiction · Alcohol · Cortico-striatal loop · Neurotransmitter · Synaptic transmission

1 Introduction

Alcoholism is a progressive and chronic relapsing disorder that ultimately leads to detrimental health outcomes. Studies have revealed adaptations to cortico-basal ganglia circuits that mediate the stages of the addiction cycle. This includes initial drug use to habitual and continued use despite negative outcomes. The transition to addiction involves neuroplasticity in these brain regions that begin in the mesolimbic dopamine region and transition to the dorsal striatum (Ito et al. 2002; Everitt and Robbins 2013, 2016). Although great progress has been made in ethanol pharmacology demonstrating that acute ethanol has only a few known primary targets (Vengeliene et al. 2008), it has long been proposed that the acute behavioral effects of ethanol are mediated principally by potentiation of γ-aminobutyric acid A ($GABA_A$) receptors and/or inhibition *N*-methyl-D-aspartate (NMDA) receptors. This presumption is due to the similarities in behavioral effects between ethanol and benzodiazepines that act on $GABA_A$ receptors, as well as NMDA antagonists such as ketamine (Krystal et al. 2003). An imbalance in the striatum of GABAergic and glutamatergic transmission is thought to play a role in alcohol use and abuse. Understanding how ethanol alters GABAergic and glutamatergic systems through the progression to alcohol addiction within specific brain regions/circuits will provide valuable insights for developing finely targeted therapeutics.

2 GABAergic Synapses

GABA is the major inhibitory neurotransmitter in the brain. GABA is derived from glutamate by the enzyme glutamic acid decarboxylase (GAD). There are two GAD isoforms, GAD65 and GAD67, named for their molecular weights. Since GAD is required for the synthesis of GABA, it is commonly used as a marker for GABAergic neurons. Once synthesized, GABA is packaged into vesicles by the vesicular GABA

transporter. The release of GABA is regulated by calcium concentration within the axon terminal, with increased concentration leading to vesicular release until basal calcium concentrations are restored.

GABA exerts its actions on transmission by $GABA_A$ ionotropic receptors and $GABA_B$ metabotropic receptors. $GABA_A$ receptors are heteropentomeric complexes that form a ligand-gated anion-selective channel that is permeable to chloride and bicarbonate. The $GABA_A$ receptor can be found both pre- and postsynaptically (Lovinger 2017). In mature neurons, the concentration of chloride is lower intracellularly leading to a net flow of anions into the neuron upon channel opening. Therefore, activation of $GABA_A$ receptors results in membrane hyperpolarization that decreases the excitability of a cell as chloride ions flow into the cell (Olsen and Sieghart 2008). In mammals, there are 19 different $GABA_A$ receptor subunits identified: α (1–6), β (1–3), γ (1–3), δ, ε, ρ (1–3), θ, and π (Olsen and Sieghart 2008, 2009). In addition to the 19 subunits, there are also subunit splice variants and phosphorylation states that can modify the activation of specific subunits. Most mammalian receptors consist of 2 α, 2 β, and 1 γ subunit. The subunits composing a $GABA_A$ receptor dictate their biophysical and pharmacological properties and location within the brain, as well as cellular distribution. Sensitivity to GABA is determined primarily by the α subunit expressed. $GABA_A$ receptors can possess a variety of allosteric modulatory sites also dependent on the subunits expressed, that allow modulators such as benzodiapines, neurosteroids, and barbiturates to alter their function. Most receptors containing the γ2 subunit are targeted to the synapse via their interaction with the scaffolding protein gephyrin (Farrant and Nusser 2005; Fritschy et al. 2012). The exception to this rule is α5βxγ2 receptors, which are targeted to the extrasynapse by the interaction of the α5 subunit and radixin (Loebrich et al. 2006). Receptors containing the δ subunit are localized exclusively in the extrasynapse (Walker and Semyanov 2008; Belelli et al. 2009; Herd et al. 2013). Posttranslational modifications of $GABA_A$ receptors regulate trafficking and stability. For example, Protein Kinase-A (PKA) phosphorylation of the β3 subunit leads to internalization of the receptor complex, whereas Protein Kinase C (PKC) phosphorylation of multiple subunits leads to membrane insertion (Kittler et al. 2005; Mele et al. 2014). Within the striatum, $GABA_A$ receptor subunits (α1–5, β1–3, γ1–3, and δ) are expressed to varying degrees, with the α2 and β3 subunits having the highest degree of immunoreactivity. The α2βxγ1/2 isoform is the most highly expressed isoform in the striatum and is located within the synaptic and extrasynaptic cellular compartments of striatal medium-sized GABAergic projection neurons (MSNs) (Schwarzer et al. 2001; Maguire et al. 2014). The α4βxδ isoform of the $GABA_A$ receptors is found exclusively extrasynaptically in MSNs and interneurons (Schwarzer et al. 2001; Maguire et al. 2014).

The $GABA_B$ receptors are Gi-protein-coupled receptors. When activated, they mediate inhibition by activating potassium channels and decreasing calcium conductance. There are two subtypes of the $GABA_B$ receptor, $GABA_BR1$ and $GABA_BR2$, that form homo- and heterodimers in the membrane. $GABA_B$ receptors can be located either pre- and postsynaptically. Presynaptic $GABA_B$ receptors serve as autoreceptors, regulating the release of GABA. Postsynaptically located $GABA_B$ receptors are primarily coupled to the activation of potassium channels (Misgeld et al. 2007), whose activation serves to hyperpolarize the cell, albeit at a slower time scale than $GABA_A$ receptors.

GABA transmission is terminated when GABA is cleared from the synapse. Reuptake of GABA is mediated by GABA transporters located on the plasma membrane of both neurons and glia. Following uptake by both cell types, GABA is degraded by the enzyme GABA transaminase into glutamate.

3 Glutamatergic Synapse

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate is synthesized by two different mechanisms: the first is synthesis from glucose through the Krebs cycle by transamination of α-ketoglutarate. Alternatively, glutamate is formed directly from glutamine by the glutaminase enzyme. Glutamate is packaged into synaptic vesicles by vesicular glutamate transporters.

The postsynaptic actions of glutamate are mediated by ionotropic and metabotropic glutamate receptors. The ionotropic glutamate receptors, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid (KA) receptors, are glutamate-gated cation channels.

NMDA receptors are voltage-sensitive ionotropic glutamate receptors that when open allow for the flow of calcium and/or sodium ions, albeit at slower kinetics than AMPA and KA receptors. NMDA receptors are regulated by presynaptic glutamate release and postsynaptic mechanisms such as phosphorylation states, subunit expression, and membrane potential that all contribute to its roles in neuronal plasticity, stabilizing neuronal activity, and coincidence detection (Malenka and Nicoll 1999; Wang 1999; Yuste et al. 1999). The voltage sensitivity aspect of NMDA receptors is due to the blockade at resting membrane potentials by magnesium, which is removed by membrane depolarization. NMDA receptors are tetramers that consist of an obligatory NR1 subunit and regulatory NR2 (A–D) and/or NR3 (A–B) subunits. The regulatory subunits control the biophysical (conductance and open probability) and pharmacological properties of NMDA receptors (Wenzel et al. 1997; Traynelis et al. 2010). NMDA receptors are unique in that they require the binding of two different ligands, glutamate and glycine/d-serine, for activation. NR1 subunits bind glycine or d-serine, while the NR2 subunits bind glutamate (Gonda 2012). NMDA receptors play a role in synaptic plasticity mainly in the form of long-term potentiation that is associated with increases in membrane insertion of AMPA receptors, protein synthesis, spine formation, and the enlargement of existing spines (Malinow and Malenka 2002; Matsuzaki et al. 2004; Kasai et al. 2010). Phosphorylation state of NMDA receptors plays a role in their localization, activation state, and physiological properties (Traynelis et al. 2010).

The AMPA and KA glutamate receptors are also heterotetrameric protein complexes that form ligand-gated ion channels. AMPA receptors consist of GluR1–4 (also known as GluRA-D), GluRδ1, and GluRδ2 (Dingledine et al. 1999). Each GluR subunit contains a binding site for glutamate. Although AMPA receptors are capable of allowing the flow of calcium, sodium, and potassium, the majority of AMPA receptors contain the GluR2 subunit which renders the ion channel impermeable to calcium. AMPA receptors mediate most of the excitatory transmission in the brain due to its vast brain expression as well as lack of voltage sensitivity (as in the case of NMDA receptors). AMPA receptors have been well studied and shown to play a role in synaptic plasticity. KA receptor subunits

include GluR5–7, KA1, and KA2 (Dingledine et al. 1999) and their activation leads to the flow of sodium and potassium ions, and consequently membrane depolarization.

Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors. mGluRs are divided into three familial groups. Group I mGluRs (mGluR1 and mGluR5) are Gq-coupled receptors whose binding of glutamate leads to activation of the enzyme phospholipase C (PLC) that ultimately induces the release of calcium from intracellular stores and increases PKC activity (Kenny and Markou 2004). Group1 mGluRs are mainly located on the postsynaptic membrane. mGluR 1 has moderate expression in the dorsal striatum and low expression in the NAc, whereas mGluR5 is highly expressed in the entire striatum (Olive 2009; Pomierny-Chamiolo et al. 2014). Group II mGlurRs (mGluR2 and mGluR3) are Gi/o-coupled receptors whose activation decreases the activity of adenylyl cyclase, ultimately decreasing the intracellular concentration of cyclic adenosine monophosphate (cAMP) (Kenny and Markou 2004). They are present both pre- and postsynaptically. Lastly, Group III mGlurRs (mGluR4, GluR6, GluR7, and GluR8) are similar to Group II mGluRs in that they are Gi/o-coupled receptors (Kenny and Markou 2004). These receptors are primarily located presynaptically and play a role in regulating neurotransmitter release. Of the Group III mGluRs, mGluR4, mGluR7, and mGluR8 are expressed in the striatum (Corti et al. 2002; Messenger et al. 2002; Bragina et al. 2015).

Similar to GABA, glutamate transmission is terminated when glutamate is cleared from the synapse. Glutamate is predominately taken up through plasma membrane transporters that are located on glia, mainly astrocytes, and to a lesser extent, on neurons. Once transported intracellularly, glutamate is metabolized to glutamine.

Corticostriatal GABAergic synapses can undergo synaptic plasticity by both long-term potentiation (LTP) and long-term depression (LTD). Corticostriatal LTP requires the activation of NMDA and D1Rs (Calabresi et al. 2000; Kerr and Wickens 2001), while LTD requires activation of postsynaptic mGluRs and presynaptic CB1 receptors (Calabresi et al. 2000).

4 The Striatum and Action Control

Cortico-basal ganglia loops play a role in the learning and selection of appropriate action sequences, as well as detecting deviations within the sequence and changes in the outcome. Cognitive control is required to guide the selection of appropriate actions based on an individual's current goals and situation, and at the same time inhibiting unwanted actions. The striatum, the main input of the basal ganglia, is innervated from all regions of the cortex. The striatum is divided into the dorsomedial striatum (DMS, roughly equivalent to the caudate nucleus in primates), dorsolateral striatum (DLS, roughly equivalent to the putamen nucleus in primates), and ventral striatum. The ventral striatum can be divided into the nucleus accumbens (NAc) and the olfactory tubercle (Heimer and Wilson 1975). Although, the striatum receives input from all regions of the cortex, specific cortical regions send inputs to distinct striatal regions. The striatum then sends converging projections to the output nuclei, the internal globus pallidus (GPi) and substantia nigra pars reticulata (SNpr). The output nuclei project to the thalamus that

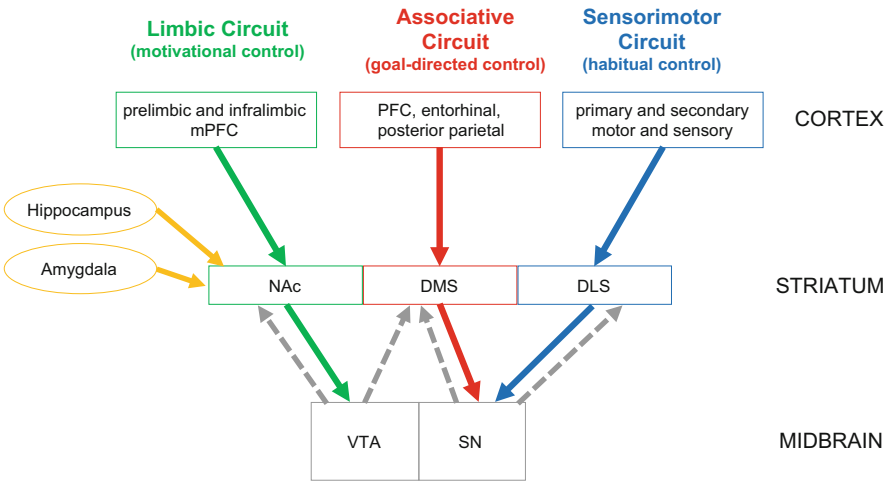

Fig. 1 Model of cortico-striatal circuitry focusing on the limbic (green), associative (red), and sensorimotor (blue) circuits. The nucleus accumbens (NAc) receives glutamatergic input from the limbic cortices, hippocampus (yellow) and amygdala (yellow), and dopaminergic projections (gray dashed lines) from the ventral tegmental area (VTA). It then projects to the VTA. The dorsomedial striatum (DMS), or the caudate nucleus in primates, receives glutamatergic input from the associative cortices and dopaminergic input from the VTA and substantia nigra (SN). It then projects to the SN. The dorsolateral striatum (DLS), or the putamen nucleus in primates, receives glutamatergic input from the sensory and motor cortices and dopaminergic input from the SN. It then projects to the SN. The VTA projects back to the NAc and the DMS, while the SN sends projections back to the DMS as well as the DLS

sends projections back to the cortical input regions, completing the loop (Alexander et al. 1986). Cortico-basal ganglia circuits can be divided based on their cortical inputs and their roles in learning, control, and performance of actions (Haber et al. 2000). These parallel circuits compete for the control of behavior and are suggested to be connected in a spiraling manner whereby MSNs in one striatal region project to the ventral tegmental area (VTA)/SN that send projections to MSNs of another striatal region, starting from the NAc medial shell then to the NAc core/DMS and ultimately to the DLS (Fig. 1). Dopaminergic innervation of the DLS is thus under the influence of the NAc.

Most alcohol users are casual consumers in which alcohol is consumed for its rewarding properties. In these individuals, alcohol consumption is thought to be a goal-directed action, in which alcohol use is dependent on the value of the outcome. Therefore, if alcohol consumption is devalued, for example it has become associated with unwanted intoxication/illness, alcohol use will cease. However with repeated, chronic use, alcohol drinking may transition to a habit in which an associated cue triggers voluntary alcohol consumption despite negative repercussions. This is highlighted by continued alcohol abuse by habitual alcoholics despite its negative outcomes (personal, social or financial). Drugs of abuse, including alcohol, are

thought to induce abnormally strong consolidation of instrumental learning mechanisms, enhancing drug seeking behavior in response to cues or contexts that are associated with the drug. This may occur by drug-induced cortico-basal ganglia circuit plasticity that reinforces the connectivity within the "spiral," leading to the recruitment of dorsal striatum circuitry proposed to underlie this transition from casual to habitual alcohol use and seeking (Koob and Volkow 2010).

4.1 Nucleus Accumbens: Motivation and Reinforcement

The limbic cortico-basal ganglia circuit is commonly referred to as the brain reward circuit (Haber 2011), with the nucleus accumbens (NAc) as its main basal ganglia input (Fig. 1 green pathway). The NAc can be divided into two subregions, the core and shell, based on MSN morphology, neurochemistry, projection patterns, and functions (Heimer et al. 1991; Zahm and Brog 1992; Meredith 1999).

The NAc core receives most of its input from glutamatergic projections from prelimbic medial prefrontal cortex (mPFC), hippocampus and amygdala (Groenewegen et al. 1999). It is also thought to be continuous with the DMS and therefore is implicated in conditioned responding, sensory motor integration, and emotional cues (Carlezon et al. 1995; Rodd-Henricks et al. 2002; Sellings and Clarke 2003; Ikemoto 2007; Guo et al. 2009; Suto et al. 2010). The NAc core sends GABAergic projections to the dorsolateral ventral pallidum and the substantia nigra (Zahm and Heimer 1990; Heimer et al. 1991; Zhou et al. 2003).

The NAc shell receives dopaminergic input from the VTA and glutamatergic projections from infralimbic mPFC, the basolateral amygdala, and ventral hippocampus (Britt et al. 2012; Papp et al. 2012). The NAc shell in conjunction with the bed nucleus of the stria terminalis and central amygdala have collectively been referred to as the extended amygdala complex, due to similarities in morphology and circuitry (Hopkins and Holstege 1978; Heimer and Alheid 1991). The extended amygdala complex has been implicated in the emotional processing of stimuli and drug addiction (Koob 2013). MSNs of the NAc shell project to the ventromedial ventral pallidum and the ventral tegmental area (Zahm and Heimer 1990; Heimer et al. 1991; Zhou et al. 2003). The NAc shell is implicated in reward processing, the control of motivation, behaviors by primary rewards, and behavioral inhibition such as aversion learning (Kravitz et al. 2012; Hikida et al. 2013).

With regards to alcohol use, ethanol increases the release of dopamine in the NAc. This increase in dopamine is believed to mediate the positive reinforcing effects of ethanol (Imperato and DiChiara 1986). The ventral striatum is also suggested to play a role in drug-induced increase in locomotion and cue-induced alcohol use.

4.2 DMS: Goal-Directed Behaviors

The DMS in the rodent, roughly equivalent to the primate caudate nucleus, is one of the subdivisions of the dorsal striatum that lines the lateral ventricle. It is part of the

associative circuit that includes the prefrontal, entorhinal, and posterior parietal cortical projections to the medial striatum (Fig. 1, red pathway). The DMS also receives dopaminergic inputs from the substantia nigra and the VTA and glutamatergic inputs from the basolateral amygdala and thalamus (Haber et al. 2000; Ikemoto 2007; Pan et al. 2010; Corbit et al. 2013; Kupferschmidt et al. 2015). MSNs of the DMS project to the substantia nigra pars reticulate, subthalamic nucleus, and globus pallidus.

As part of the associative circuit, the DMS is thought to influence goal-directed actions based on the expected consequences or value of the actions, also referred to as action-outcome (Yin et al. 2005a, b; Gunaydin and Kreitzer 2016). Goal-directed actions are flexible in that their performance is sensitive to changes in value or motivation for the outcome as well as changes in the contingency between the action and outcome.

Studies using an outcome devaluation test that examines whether a goal-directed or habitual strategy is used to perform an instrumental action (i.e. nose poke or lever press) suggest that early alcohol use is a goal-directed action (Corbit et al. 2012, 2014). In this task, rodents are trained to perform an instrumental action for alcohol. During the outcome devaluation test, the value of the alcohol reward is changed by either pairing it with an aversive stimuli, satiation to alcohol, or changing the contingency between the action and alcohol reward. With extended exposure to the action-alcohol pair, self-administration shifts from a goal-directed action sensitive to the change in the value of the alcohol reward to a value-insensitive habitual action that occurs regardless of a change in the value of the alcohol reward. (Corbit et al. 2012; Lopez et al. 2014). Pharmacological inactivation of the DMS, but not the DLS, led to a loss in the sensitivity to alcohol devaluation in mice suggesting that the DMS is important for goal-directed alcohol self-administration (Corbit et al. 2012).

4.3 DLS: Habit Formation

The sensorimotor circuit includes primary and secondary sensory and motor cortices that project to the DLS (equivalent to the primate putamen nucleus), a subdivision of the dorsal striatum (Fig. 1, blue pathway). The DLS also receives convergent dopaminergic input from the substantia nigra. The proposed role of the sensorimotor circuit is the maintenance and execution of well-learned actions that rely on external and internal cues and less on the changes in the consequences of the action often referred to in conditioning terms as stimulus-response control over behavior (Webster 1961; McGeorge and Faull 1989; Yin et al. 2006). DLS MSNs, in turn, send GABAergic projections to the substantia nigra, globus pallidus, and subthalamic nucleus.

As part of the sensorimotor cortico-striatal loop, the DLS is involved in habit formation or stimulus-response learning. A habitual behavior is an overlearned action associated with a specific cue or context. When triggered by that cue or context the habit will be performed automatically regardless of the outcome. Habitual actions require less executive control than goal-directed actions (Dalley et al.

2004; Muller et al. 2007), and therefore allows the habit system to react quickly yet it also makes it inflexible.

The habit system is thought to be strengthened under alcohol conditions in which executive control over drug taking is decreased. The DLS is also implicated in the drug-induced stereotypies as well as habitual and compulsive alcohol seeking (Everitt et al. 2008; Corbit et al. 2012, 2014). Relapse is also thought to involve the DLS (Fuchs et al. 2006). During chronic alcohol use DLS output is potentiated while the circuits involved in associative learning, such as the DMS, are altered (Belin-Rauscent et al. 2012; Hogarth et al. 2013).

4.4 Subtypes of Striatal Neurons

The striatum contains medium-sized GABAergic spiny projection neurons (MSNs), GABAergic interneurons, and cholinergic interneurons. MSNs are the principal cells of the striatum, constituting 90% of the neuronal population in rodents and 70% in primates (Kemp and Powell 1971; Kita and Kitai 1988). As the sole output neurons of the striatum, MSNs process and integrate information from cortex, thalamus, limbic structures, VTA/SN, and neighboring striatal neurons.

MSNs can be further divided into two classes based on their axonal projections, dopamine receptor expression, peptide expression, and electrophysiological properties. MSNs that express the dopamine D1 receptor, co-express dynorphin, substance P, and M4 cholinergic receptors, and directly project to the substantia nigra, are referred to as direct pathway MSNs (dMSNs) (Augood et al. 1997; Gerfen and Surmeier 2011). MSNs that express the dopamine D2 receptor, co-express enkephalin and neurotensin, and indirectly projects to striatal output regions are referred to as indirect pathway MSNs (iMSNs) (Le Moine and Bloch 1995; Surmeier et al. 1996; Augood et al. 1997; Aubert et al. 2000; Gerfen and Surmeier 2011). A small proportion of MSNs co-express D1 and D2 receptors in rodents and primates (Le Moine and Bloch 1995; Aubert et al. 2000). Direct pathway MSNs promote actions by disinhibiting the thalamus and cortex, whereas iMSNs "stop" actions by indirectly disinhibiting the SN (Kravitz et al. 2010). The balance between dMSN and iMSN activity is required for normal reward-related behaviors (Fig. 2 top). The basal electrophysiological properties of dMSN and iMSN neurons differ in the striatum, such that iMSNs are more excitable than dMSNs (Kreitzer and Malenka 2008; Grueter et al. 2010; Planert et al. 2013). Similarly, there is an increase in glutamate release onto iMSNs as compared with dMSNs (Kreitzer and Malenka 2008; Grueter et al. 2010). Although both MSN subtypes can undergo LTD including those that are NMDA-dependent, endocannabinoid-mediated, and transient receptor potential cation channel (TRPV) 1-dependent, LTD is more robust in iMSNs (Kreitzer and Malenka 2007; Grueter et al. 2010). Dopamine also leads to differential actions on dMSNs and iMSNs. When dopamine is released in the striatum, D1 receptor activation on dMSNs results in long-term potentiation (LTP) of synaptic efficacy. At the same time, D2 receptor activation prevents LTP on these synapses (Reynolds and Wickens 2002).

Although a majority of dMSNs and iMSNs are distinguished by expression and output regions, it should be noted that they are not entirely segregated. In the NAc,

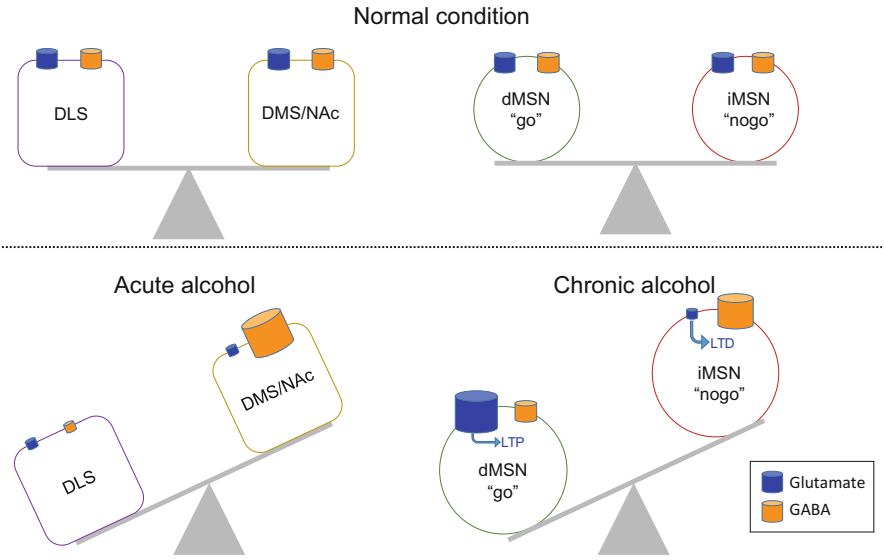

Fig. 2 Hypothetical model of the effects of acute and chronic alcohol on glutamatergic and GABAergic transmission onto the striatal subregions (left) and direct and indirect pathway MSNs of the striatum (right). Under normal conditions, glutamatergic (blue) and GABAergic transmission (orange) are balanced. This balance in output from the DLS and DMS/NAc as well as between dMSNs and iMSNs leads to normal action selection. Under acute alcohol exposure, data suggests that there is an overall hypoglutamatergic state in the striatum. However, there is a differential alcohol-induced effect on GABAergic transmission between striatal subregions: the acute ethanol-induced decrease in GABAergic transmission in the DLS and increase in GABAergic transmission in the DMS/NAc may lead to an imbalance in control of action selection between the striatal subregions. Under chronic alcohol exposure, there is a hyperglutamatergic state in dMSNs that is thought to lead to LTP of glutamatergic input onto dMSNs. In iMSNs, a decrease in NMDA receptors may lead to a decrease in glutamatergic transmission or LTD, concomitant with an increase in GABAergic transmission. Ultimately this is thought to bias towards activation of dMSNs and abnormal control over alcohol consumption

dMSNs and iMSNs are not as clearly segregated as in the dorsal striatum, with a larger proportion of MSNs co-expressing D1 and D2 receptors (Bertran-Gonzalez et al. 2008; Kupchik et al. 2015). dMSNs have been shown to send axon collaterals to the globus pallidus external and the ventral pallidum (Lu et al. 1998; Zhou et al. 2003; Fujiyama et al. 2011; Kupchik et al. 2015).

In addition to MSNs, approximately 4% of rodent striatal neurons are locally projecting GABAergic interneurons. Striatal GABAergic interneurons are medium sized, aspiny neurons and can be divided into three subtypes based on their electrophysiological properties and protein expression. Fast-spiking interneurons express the calcium binding protein parvalbumin and exhibit rapid and sustained firing (Cowan et al. 1990; Kawaguchi et al. 1995; Tepper and Bolam 2004). They provide the strongest input onto MSNs with approximately 100 connections targeting the soma and proximal dendrites of neighboring MSNs (Koós and Tepper 1999). Due to

their high degree of synchronization, vast number of connections, and proximity of synapses close to or on the soma, fast-spiking interneurons can greatly control action potential firing in MSNs (Kita 1993; Bennett and Bolam 1994; Koós and Tepper 1999; Kubota and Kawaguchi 2000; Tunstall et al. 2002; Mallet et al. 2005). Low threshold-spiking interneurons co-express a combination of neuropeptide Y, somatostatin, and nitric oxide synthase (Smith and Parent 1986). They have lower action potential firing rates than fast-spiking interneurons and exhibit plateau potentials. Synapses formed between low threshold-spiking interneurons and MSNs tend to be more apical on MSN dendrites than is true of fast-spiking interneurons. The last subpopulation of striatal GABAergic interneurons express calretinin and are considered to be low-threshold spiking interneurons (Tepper and Bolam 2004). This group of interneurons are less understood due to their limited numbers and the overlapping expression of calretinin on a subset of MSNs. MSNs receive both feed forward and lateral inhibition from GABAergic interneurons and recurrent collaterals from neighboring MSNs, respectively.

The remaining striatal neurons are large cell bodied (20–50 μm) and aspiny interneurons that release the neurotransmitter acetylcholine (Bolam et al. 1984; Smith and Bolam 1990; Wilson et al. 1990). These interneurons are tonically active and have a relatively depolarized resting membrane potential (Wilson et al. 1990; Kawaguchi et al. 1995). Due to their large size and vast dendritic and axonal fields, they are suggested to integrate and modulate synaptic connections (Kawaguchi et al. 1995). Although cholinergic interneurons and cholinergic transmission play a large role in striatal circuitry and alcohol addiction (Clarke and Adermark 2015; Gonzales and Smith 2015), the effects of ethanol on cholinergic transmission are not discussed in this chapter.

5 Ethanol Actions on GABAergic Transmission

Ethanol has long been thought to exert its effects through potentiating the GABAergic system. This is due to similarities in the behavioral effects between ethanol and benzodiazepines, such as sedation, decreases in anxiety, and ataxia. The activity of both ionotropic and metabotropic GABA receptors have been shown to be important for ethanol reinforcement and relapse to ethanol seeking (Augier et al. 2017).

5.1 Acute Actions

There has been mixed data regarding the acute effects of ethanol on $GABA_A$ receptor function. Alcohol is considered to be an allosteric modulator of $GABA_A$ receptors that enhances $GABA_A$ receptor activity by increasing the probability of channel opening or increasing agonist affinity (Nestoros 1980; Suzdak et al. 1986; Tonner and Miller 1995; Zhou et al. 1998; Welsh et al. 2009; Soyka et al. 2016). Other studies, however, found a lack of ethanol effect on $GABA_A$ receptor properties (Gage and Robertson 1985; Siggins et al. 1987; White et al. 1990). The discrepancy between studies has

been suggested to occur because of discrepant subunit composition, brain location, or phosphorylation state. In regards to subunit composition, it has been proposed that there is a specific ethanol binding site on the $GABA_A$ receptor located between the α and β3 subunit interface, or between the transmembrane region between TM2 and TM3. If this is the case, then it is safe to assume that $GABA_A$ receptors expressing a specific subunit composition profile will be sensitive to ethanol. Low concentrations of ethanol have been shown to act directly on extrasynaptic $GABA_A$ receptors containing α4/6β3δ (Wallner et al. 2006; Olsen et al. 2007), but this observation was contradicted elsewhere (Borghese et al. 2006; Botta et al. 2007). Several studies have demonstrated an involvement of PKC and its phosphorylation of subunits on the sensitivity of $GABA_A$ receptors to ethanol (Aguayo and Pancetti 1994; Weiner et al. 1994, 1997; Qi et al. 2007).

Failure to find a consistently observed direct ethanol effect on $GABA_A$ receptors suggests that the effect of ethanol on GABAergic transmission is perhaps at least partially via a presynaptic mechanism. Several studies have demonstrated an ethanol-induced increase in GABA release in various brain regions including the hippocampus, VTA, amygdala, spinal cord, cerebellum, and striatum (Crowder et al. 2002; Melis et al. 2002; Roberto et al. 2003; Ziskind-Conhaim et al. 2003; Ariwodola and Weiner 2004; Carta et al. 2004; Nie et al. 2004; Sanna et al. 2004; Siggins et al. 2005; Li et al. 2006; Ming et al. 2006; Zhu and Lovinger 2006; Criswell et al. 2008; Silberman et al. 2008; Theile et al. 2008; Wilcox et al. 2014). However not all studies have found an ethanol-induced alteration in GABA release (Proctor et al. 2006; Criswell et al. 2008). With regards to the striatum, the effects of GABAergic transmission appear to be subregion specific as it has been shown that acute ethanol increases the frequency of mIPSCs in the DMS and NAc but conversely decreases it in the DLS (Nie et al. 1997, 2000; Wilcox et al. 2014).

The regulation of GABA release by acute ethanol is thought to rely on activation of G-protein-coupled receptors including $GABA_B$ receptors (Peris et al. 1997; Nie et al. 2004; Wu et al. 2005; Silberman et al. 2009; Kelm et al. 2011). Acute ethanol has been shown to increase presynaptic $GABA_B$ receptor activity, suggesting its role in the ethanol-induced changes in GABA release. The presynaptic $GABA_B$ effect is thought to involve tonic inhibition of PKC. An ethanol-induced effect on postsynaptically located $GABA_B$ receptors has not been found (Ariwodola and Weiner 2004). The ethanol-induced alterations in presynaptic GABA release are further discussed in the chapter "Presynaptic Ethanol Actions: Potential Roles in Ethanol Seeking" found in this volume (Lovinger 2017).

5.2 Chronic Actions

Chronic ethanol exposure can result in tolerance to alcohol's behavioral effects. This may reflect neural adaptations that ultimately decrease GABAergic transmission (Fig. 2, bottom). Changes in $GABA_A$ receptor subunit expression have been found in several brain regions coincident with chronic alcohol exposure (e.g., Papadeas et al. 2001; Cagetti et al. 2003; Floyd et al. 2004; Hemby et al. 2006; Jin et al. 2014).

Specifically, in the NAc a decrease in the α4 subunit was found following 2 weeks of chronic ethanol drinking in rats compared to ethanol naïve rats (Papadeas et al. 2001). A different group demonstrated within the NAc a coordinated decrease in the protein and functional expression of the α1 and δ subunits, concomitant with an increase in α4, α5, and γ2 subunits, using a chronic intermittent ethanol exposure model followed by up to 40 days of withdrawal (Liang et al. 2014). This is suggestive of long-lasting adaptations in GABAergic transmission following chronic ethanol exposure. These differences may be due to length of exposure or withdrawal. In ethanol-dependent individuals engaged in prolonged withdrawal, lower $GABA_A$ receptor availability is found in the NAc of dependent individuals compared to controls (Lingford-Hughes et al. 2012). These adaptive changes in $GABA_A$ receptor subunit expression have also been found in other brain regions such as the hippocampus (Cagetti et al. 2003; Liang et al. 2004) and amygdala (Floyd et al. 2004; Roberto et al. 2004; Anderson et al. 2007). These alterations may induce changes in the functional properties of $GABA_A$ receptors leading to changes in affinity for GABA and allosteric modulators.

A few studies have reported that chronic ethanol exposure alters GABA release. An increase in GABA release was suggested to occur within the CeA and hippocampus of chronic ethanol treated rodents perhaps attributable to changes in the activity of $GABA_B$ autoreceptors (Tremwel et al. 1994; Peris et al. 1997; Roberto et al. 2004, 2008, 2010). Specifically, in the dorsal striatum a decrease in the frequency of mIPSCs was found in both the DLS of mice and in the putamen of monkeys (Cuzon Carlson et al. 2011, 2017; Wilcox et al. 2014). However, the role of $GABA_B$ receptors in modulating GABA release was not examined in these studies.

5.3 Pharmacotherapies for Alcohol Use Disorders That Target GABAergic Transmission

To date, there are only three medications approved by the US Food and Drug Administration (FDA) to treat alcohol use disorder: disulfiram, naltrexone, and acamprosate. Although these drugs have been shown to reduce alcohol consumption, their effects are modest and inconsistent. Therefore, there is a need to discover other pharmacological treatments need to be explored. The observed behavioral similarities between the effects of ethanol and benzodiazepines (Krystal et al. 2003) suggest that targeting the GABAergic system is a viable target for treating alcohol use disorder. There are three pharmacological agents that are thought to alter the GABAergic system that are currently being assessed for their ability to treat AUD: gabapentin, baclofen, and sodium oxybate. Gabapentin is structurally similar to the GABA, although it has no activity at GABA receptors. It is shown to have activity at voltage sensitive calcium channels and the ability to modulate GAD, increasing GABA synthesis (Taylor 1997). Gabapentin is currently FDA approved for the treatment of seizures, neuropathic pain, and restless leg syndrome. For alcohol use disorders, gabapentin has been shown to increase the rate of abstinence, decrease heavy drinking days, and decrease withdrawal symptoms (Voris et al. 2003; Mason et al. 2014). Baclofen is an agonist of the $GABA_B$ receptor and is currently FDA approved for muscle spasticity. Clinical studies have shown an increase in abstinence and a decrease in alcohol

consumption in AUD individuals using baclofen over placebo (Addolorato et al. 2007; Muller et al. 2015; Beraha et al. 2016; Reynaud et al. 2017). Sodium oxybate is the salt version of gamma-hydroxybutyrate (GHB), an endogenous neurotransmitter that has agonist activity at GABA receptors (Kamal et al. 2016). Sodium oxybate is FDA approved for the treatment of narcolepsy and is currently approved for alcohol relapse prevention in Italy and Austria. Clinical trials suggest that sodium oxybate increases abstinence rates (Gallimberti et al. 1992; Caputo et al. 2007) but may lead to craving and abuse of GHB (Caputo et al. 2007).

6 Ethanol Actions on Glutamatergic Transmission

The glutamatergic system has been implicated in the acute intoxicating effects of ethanol, ethanol dependence, and withdrawal, and recently has been suggested to be a potential target for treatment of alcoholism. These acute intoxicating effect correlates with a decrease in glutamatergic function. There is evidence that changes in brain circuitry occurring as a result of chronic alcohol exposure leads to a hyperglutamatergic state (Fig. 2). The glutamatergic system plays a role in alcohol-associated dependence, including chronic alcohol seeking and relapse (Dahchour et al. 1998; Rossetti et al. 1999; Bäckström and Hyytiä 2004; Krupitsky et al. 2007; Nagy 2008; Alasmari et al. 2015).

6.1 Acute Actions

Acute ethanol has been shown to elicit both pre- and postsynaptic effects that ultimately lead to decreased glutamatergic transmission. Presynaptically, acute low concentrations of ethanol elevate glutamate levels in the striatum (Moghaddam and Bolinao 1994; Selim and Bradberry 1996; Lominac et al. 2006; Szumlinski et al. 2007; Soyka et al. 2016; Goodwani et al. 2017; Hopf 2017) whereas at acute higher concentrations ethanol can decrease extracellular glutamate concentrations (Moghaddam and Bolinao 1994; Piepponen et al. 2002; Tiwari et al. 2014). However, other studies suggest no acute ethanol effect on glutamate levels (Dahchour et al. 1994, 1996; Quertemont et al. 2002). This discrepancy may be due to strain differences, differences in ethanol sensitivity, concentration of ethanol examined, or brain location. Nevertheless, it is proposed that ethanol can alter extracellular glutamate levels by exerting an effect on glutamate uptake by astrocytes (Smith 1997; Othman et al. 2002; Melendez et al. 2005) or by the effect of high concentrations of ethanol inhibiting the release of glutamate by its action on NMDA receptors (Martin and Swartzwelder 1992; Woodward 1994).

Postsynaptically, ethanol has been shown to alter the functioning of ionotropic and metabotropic glutamate receptors. NMDA receptor, an ionotropic glutamate receptor, is one of the major targets of ethanol (Lovinger et al. 1989, 1990; Holmes et al. 2013) and inhibition of NMDA receptors by alcohol is thought to contribute to the intoxicating effects of alcohol (Hodge and Cox 1998). Acute ethanol at concentrations that mimic intoxicating levels in humans (5–50 mM or ~23–230 mg/dL) has been shown to inhibit the function of NMDA receptors in a concentration-dependent

manner (Lovinger et al. 1989, 1990). This ethanol-induced inhibition of NMDA receptors is mediated by a decreased probability of channel opening and a decrease in mean open time (Lima-Landman and Albuquerque 1989; Weight et al. 1993).

The ethanol sensitivity of NMDA receptors is thought to be dependent on subunit composition, regional differences, phosphorylation state, and extracellular concentration of magnesium. NMDA receptors containing the NR2A or NR2B subunits are most potently affected by ethanol, with less potency for NMDA receptors containing the NR2C or NR3 subunits (Kuner et al. 1993; Yamakura et al. 1993; Masood et al. 1994; Chu et al. 1995; Mirshahi and Woodward 1995; Popp et al. 1998; Woodward 2000; Smothers and Woodward 2003). Fyn kinase, PKA, PKC, and DAARP-32 have been shown to phosphorylate NMDA receptors after ethanol administration (Moon et al. 1994; Snell et al. 1994; Miyakawa et al. 1997; Maldve et al. 2002; Li and Kendig 2003; Ferrani-Kile et al. 2003; Yaka et al. 2003) that may lead to the internalization of NR2 subunits (Suvarna et al. 2005). With regards to extracellular magnesium concentration, the inhibitory effect of ethanol on NMDA receptors correlates with the concentration of magnesium such that increasing concentrations of magnesium leads to an increase in ethanol-induced inhibition of NMDA receptors (Rabe and Tabakoff 1990; Martin et al. 1991; Morrisett et al. 1991; Calton et al. 1998). The ethanol sensitivity of NMDA receptors is also sensitive to glycine concentration, an allosteric modulator of the NMDA receptor. High concentrations of glycine (>10 μM) can decrease the ethanol-induced inhibition of NMDA receptors (Rabe and Tabakoff 1990). In the striatum, acute ethanol can inhibit the synaptic plasticity of excitatory postsynaptic currents in an ethanol concentration-dependent manner (Wang et al. 2007; Jeanes et al. 2011).

In addition to its effects on NMDA receptors, ethanol also inhibits the function of AMPA and kainate receptors (Moghaddam and Bolinao 1994; Costa et al. 2000; Crowder et al. 2002). AMPA and kainate ionotropic glutamate receptors are also sensitive to the acute effects of ethanol, albeit at concentrations exceeding those that block NMDA receptors (Lovinger et al. 1989; Dildy-Mayfield and Harris 1992; Costa et al. 2000; Moykkynen et al. 2003; Kalev-Zylinska and During 2007; Marty and Spigelman 2012; Santerre et al. 2014). A single 4-h two-bottle choice session in which mice had access to ethanol (20% v/v) and water leads to an increase in the protein level of the AMPA receptor subunit GluA1 (Beckley et al. 2016).

The role of Group I metabotropic glutamate receptors (mGluR1, mGluR5) in alcohol-related behaviors has been extensively studied. Gene variations in mGluR5 are associated with alcoholism risk (Schumann et al. 2008). Similarly, mGluR5 has been suggested to play a role in alcohol consumption and seeking as the blockade or deletion of mGluR5 specifically in the ventral striatum, attenuates these behaviors (Besheer et al. 2010; Cozzoli et al. 2012; Sinclair et al. 2012).

6.2 Chronic Actions

Chronic alcohol exposure leads to a hyperglutamatergic state in many brain regions, including the striatum (Ward et al. 2009; Ding et al. 2012, 2013; Das et al. 2015). In

response to chronic ethanol, there is a potentiation of glutamatergic transmission (Fig. 2, bottom), potentially as a compensation to chronic blockade of NMDA by acute ethanol.

In postmortem brains of human alcoholics, studies have found an increase in NMDA receptor ligand binding, density, and affinity (Michaelis et al. 1993; Freund and Anderson 1996, 1999). Similar findings were observed in rodent studies of chronic ethanol exposure in which binding to the NMDA receptor was increased concomitant with an increase in the expression of the NR1, NR2A, and NR2B subunits of the NMDA receptor (Trevisan et al. 1994; Kumari and Ticku 2000; Kash et al. 2009; Obara et al. 2009; Wang et al. 2010). In addition to up-regulation of NMDA subunit expression, chronic ethanol also increases NMDA receptor function (Kalluri et al. 1998; Carpenter-Hyland et al. 2004; Carpenter-Hyland and Chandler 2006; Kash et al. 2009; Wang et al. 2007, 2010) and conductance (Iorio et al. 1992; Sanna et al. 1993; Chen et al. 1999; Floyd et al. 2003; Nagy et al. 2003; Nelson et al. 2005). In chronic ethanol-exposed mice that were undergoing withdrawal, high frequency stimulation induced NMDA receptor-dependent LTP of glutamatergic transmission in the striatum. This was in stark contrast to the ethanol naïve condition in which the same high frequency stimulation paradigm induced NMDAR-dependent LTD of glutamatergic transmission (Yamamoto et al. 1999; Jeanes et al. 2011). The increase in LTP may be facilitated by an increase in the response of NR2B containing NMDA receptors (Wang et al. 2007). A decrease in LTD of glutamatergic transmission was also found in the striatum of chronic ethanol exposed rodents potentially through decreased endocannabinoid cannabinoid 1 receptor signaling (Xia et al. 2006; DePoy et al. 2013). These changes in NMDA receptor expression and function can lead to hyperexcitability that may underlie the increased seizure susceptibility associated with early withdrawal from ethanol (Tsai et al. 1995; Tsai and Coyle 1998). In prolonged withdrawal, a down-regulation of NMDA subunit express, function, and LTD induction have been observed in the NAc (Abrahao et al. 2013).

A chronic ethanol-induced increase in AMPA receptor expression has been found in a number of brain regions, including the striatum (Chandler et al. 1999; Neasta et al. 2010; Ary et al. 2012; Wang et al. 2012). Specifically within the striatum, an increase in the expression and synaptic trafficking of GluA1 and GluA2 subunits of the AMPA receptor was found following chronic ethanol (Neasta et al. 2010; Ary et al. 2012). An increase in AMPA receptor-mediated excitatory postsynaptic currents was observed in the DMS and amygdala following chronic ethanol (Läck et al. 2007; Ma et al. 2017). The chronic ethanol-induced facilitation of LTP observed in striatal MSNs has been suggested to involve an increase in the synaptic insertion of AMPA receptors (Wang et al. 2010, 2015).

In addition to changes in the postsynaptic ionotropic glutamate receptors, an increase in the postsynaptic release of glutamate and its concentration in brain tissue have also been observed following chronic ethanol exposure in rodents and humans (Rossetti and Carboni 1995; Bauer et al. 2013). Elevated glutamate concentrations were measured in rodents after chronic ethanol exposure in several brain regions, and the glutamate concentration measured in the NAc, in particular, correlated with

the severity of alcohol withdrawal (Fliegel et al. 2013). An increase in the spine density of MSNs as well as an increase in the frequency of excitatory postsynaptic currents were observed in the putamen of nonhuman primates (Cuzon Carlson et al. 2011). The observed chronic ethanol-induced increase in extracellular glutamate may be a result of alterations in vesicular glutamate transporters (Tsai and Coyle 1998; Fliegel et al. 2013). An increase in the expression and/or activity of mGluR1/5 may also increase glutamate release by way of activation of PLC (Obara et al. 2009; Meinhardt et al. 2013).

6.3 Pharmacotherapies of Alcohol Use Disorders That Target the Glutamatergic System

As stated above, there are currently only three FDA drugs for the treatment of AUD. Acamprosate was approved by the FDA in 2004 for the maintenance of abstinence in individuals with AUD (Maisel et al. 2013; Jonas et al. 2014; Donoghue et al. 2015). Although its exact mechanism of action is unknown, acamprosate is thought to reduce glutamate levels via antagonism of mGluR5 (Harris et al. 2003). Topiramate has shown potential as a potential AUD therapy drug. It is an antagonist of kainate and AMPA glutamate receptors (Gibbs et al. 2000) and is currently FDA approved to treat epilepsy. Topiramate has shown potential in reducing alcohol cravings and intake in human trials (Johnson et al. 2007; Blodgett et al. 2014; Martinotti et al. 2014). However, a number of adverse effects (including nausea, impaired cognitive function, and paraesthesia) has the potential to lead to noncompliance in some users as these adverse effects may outweigh the benefit of the drug (Kranzler et al. 2014).

7 Effects of Ethanol on Specific Neuronal Populations of the Striatum

Since it has been demonstrated that specific manipulation of either direct or indirect pathway MSNs leads to distinct downstream circuits mediating different behaviors (Kravitz et al. 2010; Carvalho Poyraz et al. 2016; Lambot et al. 2016), it is important to define these and other striatal cell types and highlight differences in alcohol-induced adaptations.

7.1 dMSN vs iMSNs

Evidence is mounting that GABAergic and glutamatergic transmissions within the striatum are modulated by ethanol in a cell-type specific manner. Repeated cycles of voluntary ethanol consumption and forced withdrawal selectively potentiate synaptic NMDA receptor activity in D1 receptor-expressing dMSNs but not in D2 receptor-expressing iMSNs of the DMS (Cheng et al. 2017). This imbalance in activity between dMSNs and iMSNs following chronic ethanol exposure occurs in

conjunction with a loss in the ability to elicit LTD selectively in dMSNs, while that ability was observed only in iMSNs (Fig. 2, bottom). Although this was recovered by 2 weeks of withdrawal (Jeanes et al. 2014), it is not known whether the occlusion of LTD in dMSNs following chronic ethanol was due to an ethanol-induced floor effect in LTD whereby it could not be further generated, or whether ethanol altered synaptic function in some manner rendering it resistant to change. Another study suggests that the bias towards activation of dMSNs is a result of chronic ethanol-induced facilitation of NMDA receptor currents and LTP while concomitantly inhibiting NMDA receptors and eliciting LTD in iMSNs (Fig. 2, bottom; Renteria et al. 2017). A further explanation is the increase in GluN2B-containing NMDA receptors specifically in dMSNs may facilitate AMPA receptor plasticity, LTP and an overall increase in activity of dMSNs (Wang et al. 2012, 2015).

Interestingly, this potentiation of glutamatergic transmission onto dMSNs with ethanol exposure was found to be concomitant with an increase in GABAergic transmission onto iMSNs (Fig. 2, bottom; Cheng et al. 2017). This increase in GABAergic transmission in the striatum may result from an ethanol-induced increase in GABAergic interneuron connectivity onto D2-MSNs (Gittis et al. 2011).

On the whole, the literature suggests a strong bias towards activation of dMSNs following chronic ethanol exposure that may be responsible for facilitating continued, compulsive, or excessive alcohol intake (Berglind et al. 2006; Luo et al. 2011). In agreement with an ethanol-induced increase in dMSN output, genetic knockout or blockade of glutamate receptors specifically in dMSNs that results in a decrease in output from dMSNs, reduces the alcohol deprivation effect in which there is a temporary increase in voluntary alcohol consumption over baseline when ethanol access is reinstated following a period of withdrawal (Sinclair and Senter 1967, 1968; Eisenhardt et al. 2015).

7.2 Interneurons

There are conflicting reports as to how ethanol affects fast-spiking parvalbumin expressing interneurons of the striatum. An acute ethanol-induced depression of GABAergic synapses in the DLS was found to specifically involve the GABAergic synapses of fast-spiking parvalbumin-expressing GABAergic interneurons onto MSNs through modulation of opioid transmission (Patton et al. 2016). Conversely, acute application of ethanol led to a reversible membrane depolarization in striatal fast-spiking GABAergic interneurons through a reduction in cholinergic transmission (Blomeley et al. 2011). This difference in results may have been due to species differences or differences in recording parameters. In low-threshold spiking interneurons, brief application of ethanol led to membrane hyperpolarization (Blomeley et al. 2011), an effect attributed to the ethanol's actions on potassium currents.

Acute ethanol also decreased the average action potential firing rate of cholinergic interneurons (Blomeley et al. 2011). Cholinergic interneurons have also been shown

to facilitate the acute ethanol effect on long-term synaptic plasticity in the striatum (Adermark et al. 2011).

8 Implications of the Effect of Ethanol on Striatal GABAergic and Glutamatergic Transmission in the Progression to Addiction

Repeated cycles of ethanol consumption and withdrawal are thought to reinforce ethanol consumption, in some instances leading to pathologically excessive use of ethanol. Gaining insights into the detailed mechanisms that underlie the control of ethanol consumption by excitatory and inhibitory neurotransmission onto striatal neuronal subpopulations is a required step in elucidating prospective synaptic and neuronal therapeutic targets for the development of new approaches for the treatment of alcoholism. A further goal would be uncovering differences in the cortico-basal ganglia circuits (limbic, associative, and somatosensory) to determine what sets the stage for ethanol consumption progressing to excessive, compulsive intake in some individuals while others are spared.

Acute ethanol leads to a reduction in excitatory drive by way of the inhibition of NMDA receptors. This coupled with the enhancement of inhibitory GABAergic transmission may account for the sedating and dose-dependent depressant effects of ethanol intoxication. In the striatum, the acute effects of ethanol on GABAergic transmission appeared to be subregion specific with increases in presumably the release of GABA in the DMS and NAc but a decrease in the DLS (Nie et al. 1997, 2000; Wilcox et al. 2014). This may lead to a disruption in the normal processing of reward-related behaviors mediated by the limbic circuit, or action control mediated by the dorsal striatum that may begin a bias towards the somatosensory circuit. It is posited that abnormal reward-related learning and action selection for the consumption of alcohol is brought about by ethanol-induced changes in striatal synaptic strength and plasticity. This could ultimately prime the activity of the same striatal circuits in response to future alcohol exposure. The alcohol-induced changes in neuronal signaling within the NAc could explain the erroneous cue-induced associations that are made by individuals during alcohol exposure. The activity of the DMS appears to be required during the developmental phase of excessive alcohol drinking in which the action of alcohol intake is still dependent on the outcome of that action (Corbit et al. 2012). In a related fashion, the DMS may play a role in the relapse to alcohol seeking.

One consequence of chronic ethanol exposure appears to be a disinhibition of striatal output that facilitates the recruitment of certain cortico-striatal circuits. In particular, the sensorimotor circuit may increase habit formation and alcohol seeking (Corbit et al. 2012; Dickinson et al. 2002). The resulting recruitment of the sensorimotor loop and the DLS is thought to underlie compulsive drug use. This may be further exacerbated by the alcohol-induced bias in activation of dMSNs via increased AMPAR activity. The activation or potentiation of dMSN circuitry concomitant with a decrease in iMSN circuitry could reduce the threshold of alcohol-related sensory stimulation and enhance multisensory integration surrounding alcohol use.

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The Cerebellar $GABA_AR$ System as a Potential Target for Treating Alcohol Use Disorder

David J. Rossi and Ben D. Richardson

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D. J. Rossi (✉)
Department of Integrative Physiology and Neuroscience, College of Veterinary Medicine, Washington State University, Pullman, WA, USA

Alcohol and Drug Abuse Research Program, Washington State University, Pullman, WA, USA
e-mail: david.rossi@wsu.edu

B. D. Richardson
Department of Integrative Physiology and Neuroscience, College of Veterinary Medicine, Washington State University, Pullman, WA, USA

Alcohol and Drug Abuse Research Program, Washington State University, Pullman, WA, USA

Department of Biological Engineering, University of Idaho, Moscow, ID, USA

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_109

Abstract

In the brain, fast inhibitory neurotransmission is mediated primarily by the ionotropic subtype of the gamma-aminobutyric acid (GABA) receptor subtype A ($GABA_AR$). It is well established that the brain's $GABA_AR$ system mediates many aspects of neurobehavioral responses to alcohol (ethanol; EtOH). Accordingly, in both preclinical studies and some clinical scenarios, pharmacologically targeting the $GABA_AR$ system can alter neurobehavioral responses to acute and chronic EtOH consumption. However, many of the well-established interactions of EtOH and the $GABA_AR$ system have been identified at concentrations of EtOH ([EtOH]) that would only occur during abusive consumption of EtOH ($\geq$40 mM), and there are still inadequate treatment options for prevention of or recovery from alcohol use disorder (AUD, including abuse and dependence). Accordingly, there is a general acknowledgement that more research is needed to identify and characterize: (1) neurobehavioral targets of lower [EtOH] and (2) associated brain structures that would involve such targets in a manner that may influence the development and maintenance of AUDs.

Nearly 15 years ago it was discovered that the $GABA_AR$ system of the cerebellum is highly sensitive to EtOH, responding to concentrations as low as 10 mM (as would occur in the blood of a typical adult human after consuming 1–2 standard units of EtOH). This high sensitivity to EtOH, which likely mediates the well-known motor impairing effects of EtOH, combined with recent advances in our understanding of the role of the cerebellum in non-motor, cognitive/emotive/reward processes has renewed interest in this system in the specific context of AUD. In this chapter we will describe recent advances in our understanding of cerebellar processing, actions of EtOH on the cerebellar $GABA_AR$ system, and the potential relationship of such actions to the development of AUD. We will finish with speculation about how cerebellar specific $GABA_AR$ ligands might be effective pharmacological agents for treating aspects of AUD.

Keywords

Addiction · Alcohol · AUD · Cerebellum · Ethanol · GABA

1 Introduction to $GABA_ARs$, Interactions with Alcohol, and Therapeutic Approaches to AUDs

1.1 Synaptic and Extrasynaptic $GABA_ARs$

The $GABA_AR$ is a plasma-membrane spanning, ligand-gated ionotropic channel that is primarily permeable to Cl^- (Fig. 1a) (Lorenz-Guertin and Jacob 2017). Functional $GABA_ARs$ are heteropentameric structures, comprised of varying subunit combinations from a family of 19 closely related subunit families (α1–6, β1–3 γ1–3, δ, ε θ, π, and ρ1–3; Fig. 1a, b). Most $GABA_AR$ channels in the brain are comprised of two α subunits, two β subunits, and either a γ or δ subunit, and the specific subunit makeup influences almost all biophysical/pharmacological

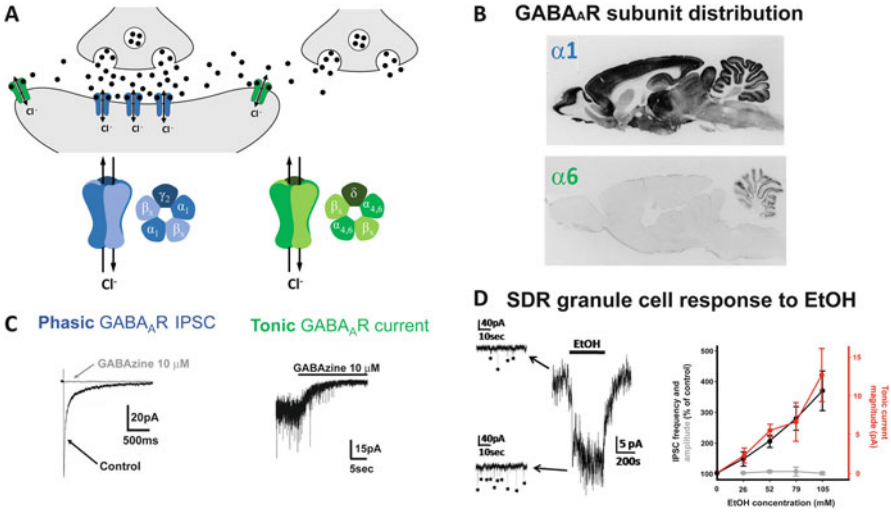

Fig. 1 Phasic and tonic GABA$_A$R currents and modulation by EtOH, as exemplified by cerebellar granule cells. (**a**) Schematic diagram showing GABA$_A$Rs in the synaptic cleft (blue) and outside of the synaptic cleft (green). Synaptic GABA$_A$Rs are typically comprised of two α subunits (with α1 dominating at most synapses), two β subunits, and a γ subunit. Extrasynaptic GABA$_A$Rs replace the γ subunit with a δ subunit which is crucial for anchoring the receptor complex extrasynaptically, and at most synapses is paired with either the α4 (hippocampus and thalamus) or α6 (cerebellum) subunit [as in (**b**), bottom panel], although other permutations also exist. (**b**) Immunocytochemistry for the α1 (top) and α6 (bottom) subunit of the GABA$_A$R receptor. **Note**, the α6 subunit is exclusively expressed in granule cells. (**c**) Phasic IPSCs (left) are mediated by synaptic GABA$_A$Rs (as evidenced by their sensitivity to the GABA$_A$R antagonist, GABAzine) that are rapidly activated by the high concentrations of vesicular GABA released into the synaptic cleft, and their decay time is dictated by receptor desensitization and inactivation as GABA is cleared from the synaptic cleft by diffusion and uptake by GABA transporters. Tonic GABA$_A$R-mediated currents (right; steady state current blocked by GABAzine; downward deflections superimposed on the tonic current are phasic IPSCs that are also blocked by GABAzine) are mediated by extrasynaptic GABA$_A$Rs that have a higher affinity for GABA and do not readily desensitize, and so generate a steady state current that varies in accordance with the concentration of ambient extracellular GABA. **Note**, because GABA released into the synaptic cleft diffuses out of the cleft where it can activate extrasynaptic GABA$_A$Rs, the magnitude of the tonic GABA$_A$R current increases or decreases in parallel with changes in vesicle release rate, either from the presynaptic neuron or from neighboring synapses not directly connected to the recorded cell. (**d**) Example voltage-clamp recording (left) showing that EtOH (52 mM) increases sIPSC frequency and tonic GABA$_A$R current magnitude in a granule cell in a slice of cerebellum from a low EtOH consuming Sprague Dawley rat (SDR). EtOH dose–response plot (right) shows the mean enhancement of sIPSC frequency (black) and tonic GABA$_A$R current magnitude (red), without affecting sIPSC amplitude (gray). Images are adapted with permission from Mohr et al. (2013) and Pirker et al. (2000)

properties of the receptor/channel complex, including channel conductance and kinetics, affinity for GABA and other agonists/antagonists, sensitivity to neuromodulators, modulation by phosphorylation, and subcellular location (for review, see Lorenz-Guertin and Jacob 2017).

Of the wide range of properties conferred by variations in $GABA_AR$ subunit composition, a major division is defined by whether their activity in situ is phasic or tonic (Fig. 1a, c) (Hamann et al. 2002; Brickley et al. 1996; Wall and Usowicz 1997; Mody and Pearce 2004; Stell and Mody 2002; Mody 2001; Lorenz-Guertin and Jacob 2017; Ye et al. 2013; Richardson et al. 2011). The nature of phasic, inhibitory postsynaptic currents (IPSCs) is dictated by the rapid activation of postsynaptic $GABA_ARs$ by transiently high concentrations of GABA (due to the proximity of synaptic $GABA_ARs$ to the vesicular release site), followed by receptor desensitization, and inactivation due to transmitter removal by plasma membrane GABA transporters (GAT1–4), which combined dictate the time course of IPSC decay (Cavelier et al. 2005; Rossi and Hamann 1998; Rossi et al. 2003; Banks and Pearce 2000; Bragina et al. 2008; Moldavan et al. 2017; Schousboe et al. 2014). In contrast, tonic $GABA_AR$-mediated currents are mediated primarily by a specialized subset of $GABA_ARs$ that are located outside of the synaptic cleft and generally have a higher affinity for GABA than synaptic $GABA_ARs$, and also are resistant to desensitization, two properties that enable extrasynaptic $GABA_ARs$ to be tonically activated by the low ambient concentration of extracellular GABA (Rossi and Hamann 1998; Hamann et al. 2002; Stell et al. 2003; Glykys et al. 2008; Cavelier et al. 2005). Specifically, tonic currents are primarily mediated by $GABA_ARs$ containing the δ subunit (rather than the more common γ subunit) combined with either the α4 or α6 subunit (depending on the brain region; Fig. 1b) which result in $GABA_ARs$ that have a high affinity for GABA, do not easily desensitize, and are anchored close to, but outside of the synaptic cleft. Importantly, although the absolute magnitude of tonic $GABA_AR$ currents is small relative to the amplitude of IPSCs, because they are constantly active, tonic inhibition is significantly more powerful than phasic inhibition, mediating ∼75% of total inhibition in cells that exhibit tonic inhibition (Richardson et al. 2011; Hamann et al. 2002). Thus, tonic $GABA_AR$ currents are potentially very powerful targets for neural modulation. Although tonic $GABA_AR$ currents have now been observed in numerous brain regions [mostly those expressing the δ subunit (Glykys et al. 2008; Richardson et al. 2011), although see (Lorenz-Guertin and Jacob 2017) for additional permutations], their properties and molecular makeup were first discovered and have been most thoroughly characterized in cerebellar granule cells (Brickley et al. 1996; Hamann et al. 2002; Stell et al. 2003; Wall and Usowicz 1997), which, given the topic of this chapter, will serve as the model for their role in the broader context of AUDs (Fig. 1c, d).

Since the ambient concentration of GABA is determined by the balance between vesicular GABA release [and possibly release from astrocytes (Rossi et al. 2003; Cavelier et al. 2005; Lee et al. 2010; Diaz et al. 2011)] and GABA removal by GATs, the magnitude of tonic $GABA_AR$ currents can be modulated by changes in (1) the rate of vesicular GABA release (whether it be into synapses on the recorded cell or from neighboring synapses not on the recorded cell), (2) the rate of GABA uptake, or (3) the density of extrasynaptic $GABA_ARs$ or their affinity for GABA (Cavelier et al. 2005; Rossi et al. 2003). Thus, changes in vesicular GABA release rate manifest as changes in the frequency of phasic IPSCs and the magnitude of tonic $GABA_AR$ currents (in cells that express relevant extrasynaptic $GABA_ARs$).

Because in most neurons in the mature brain the extracellular concentration of Cl^- is approximately 20-fold higher than the free concentration inside of cells, the reversal potential of Cl^- (E_{Cl}^-) is typically fairly hyperpolarized (−60 to −80 mV). Accordingly, activation of $GABA_ARs$ typically results in an influx of Cl^- ions, which is hyperpolarizing (i.e., inhibitory). Even if a neuron's resting membrane potential is at or near E_{Cl}^-, and thus activation of $GABA_ARs$ produces little or no current, the opening of channels and associated increase in membrane conductance may inhibit responses to excitatory inputs via shunting inhibition (Mitchell and Silver 2003; Heigele et al. 2016). This effect is more efficacious when $GABA_AR$ currents are tonically active rather than phasic, as the ability for phasic currents to cause shunting inhibition is largely dependent on their coinciding temporally (within a few ms) with the occurrence of an excitatory current. Thus, in general, any action of EtOH on the $GABA_AR$ system will enhance or reduce the primary form of fast inhibitory neurotransmission in the brain. However, in developing neurons, when the Cl^- gradient is not fully established, in subcellular compartments of mature neurons that have reduced Cl^- gradients, or in some mature neurons that have had their Cl^- gradient transiently reduced by exposure to hormones or peptides, $GABA_ARs$ may be depolarizing (Ben-Ari et al. 2012; Eilers et al. 2001; Ostroumov et al. 2016; Pugh and Jahr 2011; Tyzio et al. 2006), and thus the same action of EtOH on $GABA_ARs$ can have the opposite effect on overall cellular or subcellular excitation, although shunting inhibition can still occur even if activation of $GABA_ARs$ does depolarize a given cellular compartment.

1.2 Pre- and Postsynaptic Actions of EtOH on GABAergic Transmission

EtOH has long been known to be a potent enhancer of $GABA_AR$-mediated inhibition, but the mechanism(s) of action are complex, and vary across brain regions and $GABA_AR$ subtypes. First, in many brain regions, EtOH increases vesicular release of GABA (which can be triggered by EtOH actions in the presynaptic terminal itself, or other compartments of the presynaptic cell which induce increased action potential firing). Regardless of the site of EtOH action within the presynaptic cell that drives increased vesicular GABA release (hereafter referred to as presynaptic actions), the effect manifests as an increase in the frequency of spontaneous IPSCs (sIPSCs) and, in those brain regions that exhibit tonic $GABA_AR$ currents, an increase in its magnitude, due to spillover from the various activated synapses (Fig. 1d) (Hanchar et al. 2005; Carta et al. 2004; Liang et al. 2006; Kumar et al. 2009; Kelm et al. 2011, 2008; Criswell et al. 2008; Mohr et al. 2013; Kaplan et al. 2013). Although not the focus of this chapter, EtOH-induced changes in GABA release can also affect pre- and postsynaptic $GABA_BR$ receptors (Silberman et al. 2009; Reilly et al. 2008), which are also known to influence a variety of EtOH-related phenotypes and processes (Enoch et al. 2016; Phillips and Reed 2014), including enhancing EtOH actions on $GABA_ARs$ (Yang et al. 2000).

The ability of EtOH to increase GABA release varies across brain regions, and there is considerable variation in the underlying mechanisms across those synapses that do show EtOH-induced increased GABA release (Kelm et al. 2011). Indeed, EtOH-induced increased vesicular GABA release has been shown to be mediated by either increased action potential firing (Carta et al. 2004; Kaplan et al. 2013) or increased probability of release at the axon terminal, and the underlying molecular triggers vary from cell to cell, but often include presynaptic G-protein cascades, kinases, and a range of second messengers and effector proteins (Kaplan et al. 2013; Kelm et al. 2008, 2011; Criswell et al. 2008; Kumar et al. 2009; Nie et al. 2009).

While the evidence for EtOH increasing GABA release in various brain regions is very clear, and to our understanding not notably controversial, the more longstanding and commonly expressed thinking about EtOH actions on the $GABA_AR$ system [that it directly enhances $GABA_ARs$ (Olsen et al. 2007; Wallner et al. 2003; Hanchar et al. 2004; Crews et al. 1996; Davies 2003; Mihic 1999)] is actually far more controversial (Korpi et al. 2007; Borghese and Harris 2007; Botta et al. 2007a, b). In particular, there are certainly some clear studies showing that EtOH can increase the amplitude or decay kinetics of IPSCs and the amplitude of tonic $GABA_AR$ currents, in many cases with action potentials blocked and in the absence of any clear increase in GABA release (i.e., no change in sIPSC frequency), which is compatible with postsynaptic mechanisms (Hanchar et al. 2005; Jia et al. 2007, 2008; Liang et al. 2009). In the context of AUD, some examples of EtOH direct modulation of $GABA_ARs$ exhibit forms of adaptation to chronic EtOH exposure, which fits with a role in tolerance and dependence (Cagetti et al. 2003; Liang et al. 2006, 2009). Further, numerous studies of cloned $GABA_ARs$ expressed in isolated cell preparations have demonstrated enhancement by EtOH of responses to exogenous GABA (Meera et al. 2010). However, the existence of these presumed postsynaptic actions have been somewhat controversial. In particular, often EtOH effects vary across cell types or species despite involving apparently similar $GABA_AR$ receptor subtypes, and not all groups have observed such effects, even when studying the same preparation and cell type (see below for a more detailed discussion) (Borghese and Harris 2007; Borghese et al. 2006; Botta et al. 2007b). While this controversy has yet to be fully explained, there is accumulating evidence that the phosphorylation state of $GABA_ARs$ is a crucial determinant of whether EtOH affects postsynaptic responsivity of $GABA_ARs$ to EtOH (Choi et al. 2008; Hodge et al. 1999; Kaplan et al. 2013; Qi et al. 2007; Trudell et al. 2014).

Another concern is whether the [EtOH] required to induce direct enhancement of $GABA_ARs$ is commonly achieved in human clinical scenarios or rodent preclinical models. For example, EtOH enhancement of hippocampal and thalamic $GABA_AR$ currents doesn't occur until ~50 mM EtOH (Jia et al. 2008; Liang et al. 2009), which may be achieved in some severe cases of AUD, but is not achieved during recreational consumption in humans or in any model of voluntary consumption in rodent models. Thus, while such actions, and associated adaptations may contribute to neural processes in the late stages of AUD (Cagetti et al. 2003; Liang et al. 2006, 2009), they are not likely to play a role in initial reactions to EtOH and thus predilection and early progression to AUD. A similar concern applies to most studies of EtOH action on recombinantly expressed $GABA_ARs$ (Borghese et al. 2006).

Finally, even in cases where EtOH does alter postsynaptic $GABA_AR$ responsivity, it is not fully resolved whether such actions are due to direct interaction of EtOH with the $GABA_AR$, or whether they are secondary to phosphorylation of $GABA_ARs$ and/or $GABA_AR$ translocation to new locations within the plasma membrane or even out of the plasma membrane, all of which have also been observed in response to acute or chronic exposure to EtOH (Kumar et al. 2009; Lorenz-Guertin and Jacob 2017). Indeed, the best evidence for modulation of $GABA_ARs$ by EtOH being mediated by direct interactions comes from modeling of the ρ subunit, based on crystallographic studies of the homologous GluCl subunit (a glutamate gated chloride channel found in insects), which is an uncommon $GABA_AR$ subunit in the brain, and whose activity is actually suppressed by EtOH (Borghese et al. 2016). In this regard, we recently discovered that low [EtOH] (9 mM) can directly suppress cerebellar granule cell tonic $GABA_AR$ currents in situ, but that such suppression is prevented by postsynaptic PKC activity (Kaplan et al. 2013). Importantly in the context of AUD, the level of postsynaptic PKC activity, and thus EtOH suppression of tonic $GABA_ARs$, varies across mammalian genotypes in a manner that suggests it is a key genetically controlled, molecular determinant of excessive EtOH consumption (see below for further detail) (Kaplan et al. 2013, 2016a; Mohr et al. 2013).

Thus, while there is much evidence supporting the idea that EtOH can enhance and in some cases suppress $GABA_AR$ transmission via postsynaptic mechanisms (Kaplan et al. 2013; Borghese et al. 2016), the details of such mechanisms are far from clear. Indeed, although the $GABA_AR$ inverse agonist, Ro 15-4513, which blocks many of the intoxicating effects of EtOH has been suggested to do so by blocking EtOH binding to $GABA_ARs$ (Hanchar et al. 2006), other studies do not find any direct competitive molecular interaction between the two compounds (Korpi et al. 2007), and Ro 15-4513 activity at $GABA_ARs$ could just as well counteract EtOH intoxication by functionally counteracting enhanced GABA release, or even simply by counteracting overall changes in network activity induced by EtOH in a given brain region.

Our overall thinking on the history of and current status of direct actions of EtOH on $GABA_ARs$ is as follows. Similarities in the behavioral actions of EtOH and known $GABA_AR$ modulators (anesthetics and benzodiazepines) combined with numerous studies showing that modulating $GABA_ARs$ (pharmacologically or genetically) affects EtOH-related behavioral or even cellular phenotypes correctly led to the conclusion that a primary target of EtOH is the $GABA_AR$ "system." Parallel and/or consequent studies of recombinant $GABA_ARs$, combined with a limited number of in situ examples of EtOH modulating $GABA_ARs$ (arguably directly), refined the thinking toward the notion that a major component of EtOH actions on the $GABA_AR$ system was via direct enhancement of $GABA_ARs$. However, many of the apparent examples of direct enhancement of $GABA_ARs$ (both in situ and in recombinant systems) required higher [EtOH] than were likely involved in most behavioral actions of EtOH. Moreover, a general lack of reproducibility of some observations of direct enhancement of $GABA_ARs$, and the discovery that direct enhancement is tightly controlled by $GABA_AR$ receptor phosphorylation status,

suggests that direct enhancement may not be as common as initially thought, although it may play a role in specific cellular/behavioral situations. Further, the discovery of EtOH-induced GABA release (often at more clinically typical concentrations) combined with the fact that the dominant role of $GABA_ARs$ in all central neural processing, means that modulating $GABA_ARs$ (pharmacologically or genetically) may, and often does, alter EtOH-related behaviors even if the relevant underlying neural actions of EtOH do not involve direct enhancement of $GABA_ARs$ by EtOH at relevant concentrations. Finally, it is now clear that EtOH can actually directly suppress $GABA_ARs$, and that this process appears to be genetically regulated such that it correlates with and can drive high EtOH consumption phenotypes.

A final way in which EtOH may affect $GABA_AR$-mediated transmission is via its effect on $GABA_AR$-active neurosteroids, primarily deoxycorticosterone, progesterone, testosterone, and their respective metabolites (Helms et al. 2012; Finn et al. 2004; Porcu and Morrow 2014; Cook et al. 2014). In particular, both acute and chronic exposures to EtOH alter the local and systemic concentrations of these $GABA_AR$-active neurosteroids, either by changes in their local or global synthesis or metabolism. Often such changes vary considerably across different brain regions and across different species or genetic lines that have divergent EtOH-related phenotypes, further implicating their interaction with $GABA_ARs$ in AUDs (Jensen et al. 2017; Cook et al. 2014; Porcu and Morrow 2014; Snelling et al. 2014). It is important to note that while many such neurosteroids have been studied in the context of their ability to enhance $GABA_AR$ currents, in general the sulfated versions of otherwise $GABA_AR$-enhancing neurosteroids actually suppress $GABA_AR$-mediated currents (Helms et al. 2012; Snelling et al. 2014). Finally, it has been demonstrated that some neurosteroids can act on presynaptic GABAergic terminals to increase vesicular GABA release (Park et al. 2011). The concentration of neurosteroids required to induce such release is generally higher than the usual range detected in plasma, but it is conceivable that local neurosteroid synthesis could result in higher concentrations locally that could affect presynaptic GABA release. Regardless, potential AUD-related treatment options involving neurosteroid-$GABA_AR$ interactions could involve this process. Thus, regulation of $GABA_AR$ transmission by neurosteroids is complex on its own, and varied modulation by EtOH across different brain regions, EtOH-contexts (low versus high concentrations and acute versus chronic exposure), and species/genotypes adds another level of complexity for which considerably more research will be required to fully understand.

1.3 Preclinical Studies That Target $GABA_ARs$ to Deter EtOH Consumption Have Not Translated into Clinical Treatment for AUDs

Because of the clear interactions of EtOH with $GABA_AR$-mediated transmission, modulation of $GABA_ARs$ has been a dominant focus of preclinical efforts to combat

EtOH actions in a manner that might reduce AUDs. Such efforts tend to focus on blocking or replacing acute EtOH actions on $GABA_ARs$ and on ameliorating $GABA_AR$-related adverse reactions to chronic EtOH consumption and associated withdrawal (Anton et al. 2014). Specifically, in an early study, systemic administration of a $GABA_AR$ agonist (THIP/Gaboxadol) or antagonist (picrotoxin) increased and decreased, respectively, ongoing voluntary EtOH consumption by rats (Boyle et al. 1993). Similarly, knocking out specific $GABA_AR$ subunits globally generally reduces EtOH consumption across a range of EtOH consumption models and lines of mice (Crabbe et al. 2006b; Rewal et al. 2009, 2012; Nie et al. 2011). However, other studies have shown that systemic application of $GABA_AR$ agonists, including THIP, can reduce EtOH consumption, including binge EtOH consumption (Moore et al. 2007; Ramaker et al. 2012). Furthermore, detailed temporal analysis of EtOH bout patterns indicate that the effect of modulating $GABA_ARs$ globally, with the synthetic $GABA_AR$-enhancing neurosteroid ganaxolone, can be complex even within a single model and set of animals (Ramaker et al. 2011). Such variability with systemic application of $GABA_AR$ ligands likely reflects the widespread expression of $GABA_ARs$ across the brain, with different brain regions playing different roles in various aspects of EtOH-induced responses (Ramaker et al. 2015; Kaplan et al. 2016b; Nowak et al. 1998; Nie et al. 2011; Pina et al. 2015; Rewal et al. 2009). However, even within a given brain region, such as the nucleus accumbens, the role of $GABA_ARs$ in EtOH consumption is complex, with either blocking or activating extrasynaptic $GABA_ARs$ able to reduce EtOH consumption (Rewal et al. 2009; Nie et al. 2011; Ramaker et al. 2015). An additional complication is that because $GABA_ARs$ are widely distributed in most brain regions, many $GABA_AR$ modulators cause intolerable side effects, such as sedation, depression, and motor impairment, which preclude clinical use. Similarly, various $GABA_AR$ modulators have their own addictive potential. Thus, it is perhaps not surprising that despite the clear role of $GABA_ARs$ in multiple aspects of EtOH responses, there are currently no $GABA_AR$ ligands that are clinically effective at reducing AUDs. Instead, clinically, the use of $GABA_AR$ modulators is primarily restricted to emergency care, in particular preventing life threatening EtOH withdrawal symptoms, and there is still inadequate pharmacotherapy for AUD treatment and recovery generally (Anton et al. 2014; Eastes 2010).

1.4 The Cerebellar $GABA_AR$ System May Provide a Missing Piece to the AUD Puzzle

A likely contributing factor to the inadequate clinical translation of the aforementioned preclinical studies of EtOH and the $GABA_AR$ system is that much of what has been learned about their interactions is based on the studies of relatively high [EtOH] (almost exclusively $\geq$20 mM, and often $\geq$40 mM). While such [EtOH] are achieved in the blood of humans after binge EtOH consumption by nonalcoholics, and may be common in advanced cases of AUD, such concentrations are not achieved by humans in their early experiences with recreational EtOH consumption. This

discrepancy is problematic because individual variation in the sensitivity of various neural processes to low [EtOH] is a significant predictor of risk for developing AUD. Specifically, low sensitivity to the aversive effects and high sensitivity to the rewarding effects of low [EtOH] predict an increased risk for developing AUD (Crabbe et al. 2010; Quinn and Fromme 2011; Schuckit 1985; Schuckit and Smith 1996; Schuckit et al. 1996, 2003). Importantly, low [EtOH], such as those achieved when an adult human consumes 1–2 standard units of alcohol over a 1–2 h period (i.e., blood [EtOH] = ~10 mM), have profound impacts on affect, mood, and behavior. Effects include a sense of euphoria, social disinhibition, anxiolysis, and motor impairment (Gallaher et al. 1996; Gilman et al. 2008; Schuckit 1985; Schuckit et al. 2003, 2008; Spanagel 2009; Trudell et al. 2014). These behavioral manifestations are reflected by brain imaging studies showing low [EtOH] alters neural signaling in brain regions involved in executive function (prefrontal cortex), reward and anxiety (ventral tegmental area, striatum, and amygdala), and motor coordination (cerebellum) (Mitchell et al. 2012, 2013; Gan et al. 2014; Weber et al. 2014; Nikolaou et al. 2013a, b; Bjork and Gilman 2014; Gilman et al. 2008; Volkow et al. 2008). However, there is a significant gap in our understanding of specific cellular/molecular targets of low [EtOH], and the mechanisms by which they alter neural processing to influence behavior. In fact, although studies of isolated brain tissue have shown that higher [EtOH] (>20 mM) alter neuronal and synaptic function, the effects of low [EtOH] ($\leq$ 10 mM) at this level have been reported to be minimal or absent (Choi et al. 2008; Jia et al. 2007, 2008; Liang et al. 2006; Morikawa and Morrisett 2010; Nie et al. 1994, 2000; Peris et al. 1992; Roberto et al. 2003; Theile et al. 2008, 2009; Weitlauf and Woodward 2008). Thus, it is not clear how low [EtOH] has such robust action on cognition, emotion, and behavior, and thus how variations in those actions contribute to risk for developing an AUD. Accordingly, a primary goal of the alcohol research field has become to identify the molecular mechanisms by which low [EtOH] alters neural processing and EtOH-associated subjective effects and behaviors, and to determine how individual differences affect risk for developing AUD.

In this context, the $GABA_AR$ system of the cerebellum is an appealing target. In particular, in studies of humans and animals, low [EtOH] clearly alters cerebellar neural processing and associated behaviors (Volkow et al. 2008; Gallaher et al. 1996; Schuckit 1985; Schuckit et al. 2003). Similarly, studies of cerebellar brain slices from low EtOH consuming Sprague Dawley rats have shown that 10 mM EtOH powerfully enhances $GABA_AR$-mediated inhibition of cerebellar granule cells (Fig. 1d) (Botta et al. 2007a; Carta et al. 2004; Hanchar et al. 2005; Kaplan et al. 2013), which are the primary integrators of afferent information to the cerebellar cortex (Fig. 2). In the remainder of this chapter we will review recent advances in our understanding of the cerebellum and its potential relationship to AUDs, with a particular focus on the granule cell $GABA_AR$ system (including $GABA_ARs$ and upstream mechanisms that affect GABA release).

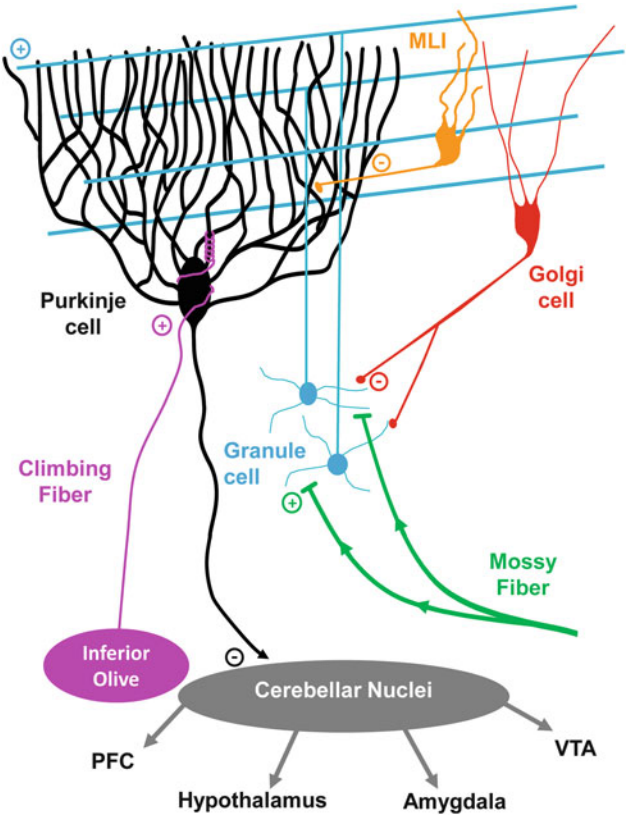

Fig. 2 Circuitry of the cerebellar cortex. Circuit diagram of the cerebellum, showing the two excitatory/glutamatergic afferent inputs to the cerebellar cortex (mossy fibers and climbing fibers), the connectivity of the interneurons, which include the glutamatergic granule cells and GABAergic Golgi cells and Molecular Layer interneurons (MLIs), and the sole output of the cerebellar cortex, the GABAergic Purkinje cells. The Purkinje cells synapse onto a variety of cells distributed into three cerebellar nuclei, which in turn send mono- and polysynaptic efferents to most of the rest of the brain

2 Introduction to and Review of the Cerebellum and Its Relationship to AUDs

2.1 The Cerebellum and Genetic Risk for AUD

The importance of the cerebellum in motor control and balance has been known for nearly 200 years (Schmahmann 2010). However, over the last two decades it has been thoroughly established that the cerebellum plays a critical role in cognitive processes that had hitherto been overshadowed by its more obvious role in motor coordination. Compelling evidence from cerebellar specific lesion (Levisohn et al.

2000; Paulus et al. 2004; Schmahmann 2004; Tavano et al. 2007; Wolf et al. 2009), functional imaging (Stoodley and Schmahmann 2009, 2010; Stoodley et al. 2010, 2012), and anatomical tracing studies (Strick et al. 2009) indicate that the cerebellum contributes to attention, executive function, visual-spatial cognition, language, and emotion through reciprocal loops to and from the association areas of the parietal, frontal, temporal, and limbic cortices (Fig. 2) (Ito 2008; Schmahmann 2010; Strick et al. 2009). The cerebellum also communicates (see below for further details) with brain regions associated with EtOH reward [ventral tegmental area (Ikai et al. 1992, 1994), amygdala (Tomasi and Volkow 2011), and nucleus accumbens (Dempsey and Richardson 1987)], and with consummatory behavior (hypothalamus (Zhu et al. 2006; Zhu and Wang 2008)).

Adoption and twin studies suggest that predilection for developing AUD is 50–60% genetically determined (Hasin et al. 2007; Hill 2010). However, it is clear that there isn't a single or even group of "AUD gene(s)," but rather that a wide range of genes lead to complex traits, and interactions amongst such traits engender a predilection for AUD. Consequently, the field of AUD researchers has tended to identify and characterize endophenotypes (genetically and mechanistically simpler heritable traits associated with genetic risk for developing an AUD), with the idea that understanding the molecular/genetic and neural substrates of such endophenotypes will be a more feasible approach to identifying potential targets for treatment of AUD. A common approach to identifying such AUD-related endophenotypes is to quantify differences in a given physical or behavioral trait between people with and without a family history of AUD (FH^+ and FH^-, respectively). Such studies have consistently identified cerebellar-related anatomical, neurological, and behavioral endophenotypes for which variation is tightly linked with AUD FH status.

First, in a series of MRI studies, Hill and colleagues determined that the cerebellum of FH^+ offspring is significantly larger than in trait matched FH^- offspring, due primarily to increased grey matter, potentially due to reduced synaptic pruning during development (Hill et al. 2007, 2011, 2016; Hill 2010). Importantly, this difference is separable from effects related to prenatal exposure to EtOH, which also affects the size of the cerebellum, but in the opposite direction and in distinct lobes (Sharma and Hill 2017). Also of interest, given the importance of $GABA_A$Rs in EtOH actions, increased cerebellar volume in FH^+ individuals is associated with an allelic variation in the $GABA_A$R α2 subunit (Hill et al. 2011), which when knocked out in mice results in reduced EtOH consumption in females (Boehm et al. 2004).

In terms of cerebellar processing and communication with other brain regions, there are also considerable differences that correlate with AUD FH status. In a series of functional connectivity magnetic resonance imaging (fcMRI) studies, Nagel and colleagues determined that relative to FH^- individuals, alcohol naïve FH^+ offspring showed less functional connectivity between the cerebellum and two brain regions known to be involved in addictive behaviors, the prefrontal cortex (PFC) and nucleus accumbens (Cservenka et al. 2014; Herting et al. 2011). Further, using functional MRI, they also determined that FH^+ individuals show reduced cerebellar

activity during cognitive tasks (risky decision making and spatial working memory), despite not exhibiting any deficits in task performance (Cservenka and Nagel 2012; Mackiewicz Seghete et al. 2013). Thus, in addition to exhibiting reduced communication with addiction-associated brain regions, alcohol naïve, FH^+ individuals exhibit altered cerebellar processing of behavioral tasks that likely play a role in addiction.

Another way in which genetic variation in cerebellar processing may influence predilection to AUD is via its role in neurological diseases/conditions that are risk factors for developing an AUD, possibly through self-medication with EtOH. The clearest example of self-medication with EtOH for a known cerebellar disease is a condition known as essential tremor, in which genetically determined cerebellar dysfunction leads to uncontrollable shaking, most frequently in the hands, but also in other body parts (Louis et al. 2017; Kuo et al. 2017). It is well established that consumption of EtOH ameliorates such tremors, and that patients use EtOH to self-medicate the condition (Rautakorpi et al. 1983; Mostile and Jankovic 2010). However, while some studies have suggested that essential tremor is a risk factor for AUD, others have not (Deik et al. 2012; Schroeder and Nasrallah 1982; Koller 1983). Similarly, tremor is a symptom of severe EtOH withdrawal, which also has a cerebellar etiology, and self-medication of the negative withdrawal symptoms may also contribute to the maintenance of AUD (Welsh et al. 2011; Deik et al. 2012).

Attention deficit hyperactivity disorder (ADHD) and schizophrenia are two genetically influenced disorders that have a strong cerebellar component to their etiology (Bledsoe et al. 2009; Mulder et al. 2008; Epstein et al. 2007; Mothersill et al. 2015; Baumann et al. 2015), and both conditions are risk factors for developing AUD (Daurio et al. 2017; Jones et al. 2011). In the case of ADHD, the relationship between risk for developing AUD is most tightly connected to the impulsivity aspects of ADHD (Daurio et al. 2017), which fits with the studies described above showing that AUD FH^+ individuals exhibit reduced cerebellar processing during tests of impulsivity, i.e., risky decision making.

As discussed above, one consistent component to genetic risk for developing an AUD is increased sensitivity to the rewarding aspects of EtOH and reduced sensitivity to the aversive aspects of EtOH. One commonly studied manifestation of sensitivity to EtOH in humans is alcohol-induced static ataxia, which manifests as body sway, resultant from impaired vestibular and ocular feedback control of balance (a process that heavily depends on the cerebellum). Notably, studies of EtOH-induced body sway consistently find that the level of sensitivity to EtOH-induced body sway is heritably associated with AUD family history status and predictive of development of AUDs (Schuckit et al. 2005, 2011; Newlin and Thomson 1990; Newlin and Renton 2010). However, the nature of the relationship is complicated, with some studies finding a low level of response to EtOH-induced body sway in FH^+ individuals, but others finding a high level of response in FH^+ individuals (Newlin and Renton 2010; Quinn and Fromme 2011; Schuckit et al. 2005; Lex et al. 1988; Newlin and Thomson 1990; McCaul et al. 1991). These distinctions may result from differences in methodology or time point after EtOH exposure, or they may reflect two distinct sets of phenotypic risk (Quinn and

Fromme 2011). This covariation also consistently occurs in animal models, albeit similar to humans, with varying polarity across studies. In particular, sensitivity to EtOH-induced ataxia shows an inverse relationship with EtOH consumption in some inbred strains of mice [e.g., DBA/2J (D2) and C57BL/6J (B6) mice] (Gallaher et al. 1996; Yoneyama et al. 2008) and lines of rodents selected for differences in alcohol consumption (e.g., Alko, Alcohol [AA]/Alko, Non-Alcohol [ANA] rats, and alcohol preferring (P)/alcohol non-preferring (NP) rats) (Bell et al. 2001, 2006; Malila 1978). Conversely, a recent study in high- and low-alcohol preferring mice (HAP and LAP) showed a positive relationship between EtOH consumption and sensitivity to EtOH-induced ataxia (Fritz et al. 2012). Thus, collectively, although the direction of the relationship varies across studies, there is nonetheless a consistent genetic relationship between cerebellar sensitivity to EtOH and risk for AUD in humans and level of EtOH consumption in rodent genotypes. In this context, it is also important to reiterate that although static ataxia in humans and rotorod performance in rodents are easily quantified measures of cerebellar sensitivity to EtOH, they do not have to be the only aspect of altered cerebellar processing that promotes or deters EtOH consumption and thus escalation to AUD. Instead, the relationship between cerebellar sensitivity to EtOH (quantified by measures of static ataxia and rotorod performance) and AUD risk could be mediated by cerebellar-dependent motor processes, cognitive processes, reward processes, or some combination. Thus, while motor impairment and disrupted balance are likely aversive (Damji et al. 1996; Hotson 1984; Ragge et al. 2003), whether or not the overall cerebellar response to EtOH promotes or deters excessive EtOH consumption likely depends on how motor aspects combine/interact with EtOH effects on the other, non-motor aspects of cerebellar processing.

In summary, there is a clear and consistent heritable relationship between genetic predisposition for developing an AUD and a variety of aspects of the cerebellum, including: (1) its size and white matter to grey matter ratios, (2) its communication with reward-associated brain regions (see below for further details), (3) its processing of behaviors that likely influence predilection for AUD, and (4) its sensitivity to EtOH. There are also a number of known cerebellar-related diseases that are also known risk factors for developing an AUD, and at least some of them are likely to provoke self-medication with EtOH.

2.2 The Neural Circuitry of Cerebellar Interactions with AUD-Associated Brain Regions

In order to consider how variable effects of EtOH on cerebellar cortical processing might influence EtOH consumption, we need to consider the outputs and function of the cerebellum. Purkinje cells are the sole output of the cerebellar cortex (Fig. 2), and they form GABAergic inhibitory synapses onto the glutamatergic/GABAergic/glycinergic neurons of the three cerebellar nuclei: the dentate (lateral), interpositus (intermediate), and fastigial (medial) (Ito et al. 1970; Billard et al. 1993; Jahnsen 1986; ten Bruggencate et al. 1972; Teune et al. 1998; De Zeeuw and Berrebi 1995;

Chen and Hillman 1993; Monaghan et al. 1986). Efferents from these nuclei then project, directly and polysynaptically, to multiple other brain regions that are important for driving and/or regulating motor output, including motor cortex, thalamus, basal ganglia, red nucleus, inferior olive, and spinal cord. This dominant efferent distribution pattern, combined with the fact that cerebellar neurons respond to movement or changes in limb/body position (Barmack and Yakhnitsa 2008; Lisberger and Fuchs 1978; Thach 1968, 1970), and that damage to the cerebellum often results in motor control abnormalities like ataxias, dysmetria, or gaze control disorders (Schmahmann 2004), demonstrate that the cerebellum serves an important role in motor control and vestibular reflexes, including balance and ocular stabilization. Importantly, EtOH-induced ataxia and motor incoordination are largely mediated by EtOH actions in the cerebellar cortex that lead to disruption of cerebellar output to these motor areas (Dar 2015; Hanchar et al. 2005).

In addition to its well-established role in motor control, functional imaging studies in humans clearly indicate that changes in cerebellar activity are correlated with numerous non-motor behaviors, including various cognitive and emotional processing tasks (Ferrucci et al. 2012; Schraa-Tam et al. 2012; Stoodley and Schmahmann 2009, 2010; Stoodley et al. 2010, 2012). In parallel animal studies, clear mono- or polysynaptic functional connections between the cerebellum and non-motor brain regions have been identified (Harper and Heath 1973, 1974; Strick et al. 2009; Zhang et al. 2016). Accordingly, selective damage of the cerebellum in humans can result in non-motor, cognitive, or emotional processing abnormalities that may present with, or even without, motor function disruption (Schmahmann and Sherman 1998; Schmahmann 2004; Schmahmann et al. 2009). These studies indicate that the cerebellum is involved in many aspects of cognition, emotion, and overall behavior (Ito 2008; Schmahmann 2004, 2010; Schmahmann and Caplan 2006).

In the context of AUDs, some of the mono- and polysynaptic projections from cerebellar nuclei neurons target brain regions known to be involved in addiction and drug abuse, including the ventral tegmental area (VTA), nucleus accumbens, amygdala, PFC, hippocampus, and hypothalamus (Tomasi and Volkow 2011; Strick et al. 2009; Volkow et al. 2008). Therefore, EtOH-induced changes in the activity of these cerebellar efferents due to modulation of cerebellar cortical activity may be a mechanism by which EtOH alters reward processing, affect, memory, or consumption behavior. Thus, it is important to consider the nature of these connections in order to understand the direction and degree to which EtOH actions in the cerebellar cortex may alter synaptic activity and firing dynamics of neurons in these other brain regions.

The collection of dopaminergic neurons within the ventral tegmental area has long been known to be important in mediating reward responses or pleasurable feelings associated with specific stimuli (e.g., food, sex, drugs of abuse) by releasing dopamine in the nucleus accumbens of the ventral striatum (Koob and Volkow 2010). Unfortunately, the mechanisms by which EtOH at doses or concentrations that are within a range commonly experienced during recreational consumption (blood [EtOH] $\approx$ 45–150 mg/dl, 10–35 mM) modulate dopamine release through

altering VTA dopaminergic neuron activity and dopamine release are unclear. In rodent models in vivo, low doses of systemically administered EtOH (0.5–2 g/kg ≈ 50–200 mg/dl) reduce firing of GABAergic (Steffensen et al. 2009; Stobbs et al. 2004) neurons and increase firing of dopaminergic neurons (Gessa et al. 1985; Ostroumov et al. 2016) in the VTA, but this approach cannot distinguish between sites of action for EtOH that are within the VTA or on VTA afferents, or on the cell bodies or synaptic inputs of such afferents. When directly administered into the posterior VTA, rats selectively bred to prefer EtOH will self-administer 50–75 mg/dl (11–16 mM) EtOH (Gatto et al. 1994; Hauser et al. 2011; Rodd et al. 2005). Although, in rat strains not bred selectively to prefer EtOH, 200 mg/dl (43.4 mM) appears to be the threshold dose to induce dopamine release in the nucleus accumbens (Ding et al. 2009), ventral pallidum, or PFC (Ding et al. 2011), and trigger self-administration (Rodd-Henricks et al. 2000). Importantly, EtOH in ex vivo brain slices typically needs to reach >40 mM before inducing any significant change in the activity of VTA dopaminergic neurons or synaptic activity in mice (Avegno et al. 2016; Brodie and Appel 2000; Okamoto et al. 2006) and rats (Xiao et al. 2009; Theile et al. 2011; Ostroumov et al. 2016; McDaid et al. 2008; Koyama et al. 2007; Brodie et al. 1990), although Mrejeru et al. (2015) recently indicated that a limited subset of VTA dopaminergic neurons may be sensitive to 20 mM EtOH. Together, these data suggest that VTA sensitivity to recreational levels of EtOH is likely not mediated entirely by mechanisms within the VTA, but rather may be dependent on modulation of VTA afferent activity from other brain regions that are only functionally intact in vivo and may induce excitation of VTA dopaminergic neurons.

One possible brain region that could mediate EtOH's effects in the VTA may be the cerebellum, since it is exquisitely sensitive to low [EtOH] (see above). A functional synaptic connection between cerebellar nuclear neurons and the VTA was demonstrated by studies in which electrical stimulation of the dentate nucleus or Purkinje cells caused increased dopamine levels in the nucleus accumbens (Dempsey and Richardson 1987) or PFC in a manner that was blocked by local pharmacological inhibition of the VTA (Rogers et al. 2011, 2013; Mittleman et al. 2008). The as yet poorly understood cerebellar efferent pathway to VTA that increases dopamine release in the PFC, nucleus accumbens, and amygdala (Inglis and Moghaddam 1999; Oades and Halliday 1987; Loughlin and Fallon 1983; Beckstead et al. 1979; Fallon et al. 1978) may couple with the activity of a direct projections from the fastigial nucleus to the nucleus accumbens and amygdala which have been demonstrated in primate, cat, and rat (Harper and Heath 1973; Heath and Harper 1974; Oades and Halliday 1987). While these pathways are poorly understood, their clear existence provides a potential substrate for the translation of EtOH-induced changes in cerebellar cortical activity into EtOH-related modulation of reward and emotional processing.

Of the dopamine-related cerebellar efferent pathways, those that influence dopamine release in the PFC, a region involved in cognition, attention, and affect, is most thoroughly characterized. Cerebellar cortical or nuclear stimulation increases PFC dopamine, which peaks within 350–400 ms poststimulation (Mittleman et al. 2008).

Specific nuclei/region inactivation studies using local application of lidocaine (a voltage-gated sodium channel blocker) or kynurenate (an ionotropic glutamate receptor antagonist) directly into the VTA alone reduced cerebellar stimulation-induced PFC dopamine levels by ~50%. Thus, half of the cerebellar-evoked PFC dopamine release is via excitation of the VTA. Further efforts to map the efferent pathway that drives such VTA excitation, again via local inactivation studies of potential intervening nuclei, revealed a dentate-reticulotegmental-peduncolopontine-VTA-PFC pathway (Rogers et al. 2011). Additional studies, blocking action potentials and glutamatergic transmission in putative complementary pathways (mediodorsal or ventrolateral thalamus) revealed that, together, pathways involving the thalamic nuclei mediate the remaining ~50% of the rise in PFC dopamine (Rogers et al. 2011). Collectively, these data suggest that electrical stimulation of the cerebellar cortex or dentate nucleus increases PFC dopamine release via parallel polysynaptic pathways that excite the VTA (dentate-reticulotegmental-peduncolopontine-VTA-PFC pathway) as well as VTA terminals (mediodorsal or ventrolateral thalamus) within the PFC (Rogers et al. 2011). However, recent exciting functional tract tracing studies have confirmed an older tract tracing study (Perciavalle et al. 1989) showing that there is also a direct projection from the cerebellar nuclei (dentate and interpositus) to the VTA (Kamran Khodakhah, personal communication; Richardson and Rossi, unpublished observations), but the function, behavioral relevance, and potential role of this pathway in mediating VTA responses to EtOH and reward have yet to be examined.

The increase in PFC dopamine levels upon cerebellar stimulation indicates the ability of dentate nuclei efferents to influence activity of both the VTA and the PFC (Rogers et al. 2011; Mittleman et al. 2008), but additional parallel cerebellar nuclear efferent pathways may also influence the PFC, albeit presumably in different contexts and time scales. In particular, electrical stimulation of the dentate nucleus evokes excitatory field potentials in the PFC in primates (Sasaki et al. 1979), and concurrent antero- and retrograde tracing techniques indicated that such potentials are mediated by dentate to thalamic nuclei to PFC pathways (Kelly and Strick 2003; Middleton and Strick 1994, 2001). Conversely, electrical stimulation of the fastigial nucleus typically induces a suppression or biphasic inhibition/excitation response of putative PFC pyramidal cells, indicating that fastigial efferents may primarily influence inhibitory synaptic activity in PFC (Kelly and Strick 2003; Middleton and Strick 1994, 2001; Watson et al. 2014), but does so after a much longer latency (10–13 ms) relative to dentate-evoked responses, suggesting that the connection is polysynaptic (Watson et al. 2014).

Thus, in addition to dentate driven VTA-derived dopamine release into the PFC, inhibitory fastigial and excitatory dentate projections to the PFC offer a mechanism by which differential EtOH-induced changes in cerebellar cortical output may be able to enhance and/or suppress PFC activity, although further work is needed to confirm the precise neuronal types that these cerebellar nuclei projections target to fully understand how the cerebellum may modulate or drive PFC activity to affect EtOH consumption.

In addition to cerebellar modulation of reward circuitry influencing the initial responses to EtOH, another way in which actions of EtOH in the cerebellum may influence abusive EtOH consumption is via an influence over cues and contexts associated with EtOH during the addiction cycle. In particular, learned associations between cues related to drugs of abuse (including EtOH) and pleasure are a key aspect of drug abuse and relapse. And, fMRI studies in abstinent alcoholic humans have shown that the cerebellum is strongly activated by cue-induced craving, and that such cue-induced activation of the cerebellum ceases to occur after cognitive therapy eliminates craving associated with the cues (Schneider et al. 2001). And a recent study in rodents found that a subset of granule cells in the cerebellar cortex is activated by cues that predict reward in a manner that suggests that they code expectation of reward (Wagner et al. 2017).

How might the cerebellum influence cue-induced craving and anticipation of reward? Given the role of the hippocampus in learning and memory, it has long been considered that the hippocampus is a key player in maintaining this association despite also contributing to AUD-associated cognitive impairment (Kutlu and Gould 2016), and evidence suggests that the cerebellum may powerfully influence the hippocampus, providing a potential mechanism by which the cerebellum influences craving for EtOH. For the purposes of this discussion, only the potential role of the cerebellum in mediating the association between context and reward will be discussed, not the multitude of deleterious effects EtOH has on hippocampal anatomy and function. Similarly, since the full range of pathways that may constitute polysynaptic connections between the cerebellum and hippocampus which shape aspects of spatial and temporal processing are vast, full coverage is also beyond the scope of this book chapter, but have been recently reviewed elsewhere (Yu and Krook-Magnuson 2015).

While there does not appear to be a direct connection between the dentate nucleus and hippocampus (Heath et al. 1978), field potential, single unit responses, and degenerating fiber tracing data from primate, cat, and rat indicate that there is a robust short latency bilateral direct projection from the fastigial nuclei to hippocampus (Newman and Reza 1979; Heath and Harper 1974; Heath et al. 1978). In line with the known expression of glycinergic projection neurons in the fastigial nucleus (Bagnall et al. 2009), this projection appears to be largely inhibitory (Heath and Harper 1974). However, these fastigial-evoked field potentials tended to be biphasic and also had a later excitatory component and generated action potentials within 12 ms of stimulation, likely due to a rebound from inhibition or activation of an additional long latency excitatory projection (Heath and Harper 1974; Newman and Reza 1979). This cerebellar-hippocampal projection is robust enough to block epileptiform activity when Purkinje cells are driven by activation of channelrhodopsin (Krook-Magnuson et al. 2014). PKC-dependent plasticity at the parallel fiber to Purkinje cell synapse in mice is essential for accurate coding of hippocampal place cells and performance on a navigation task, indicating the importance of cerebellar input to the hippocampus in maintaining spatial orientation (Rochefort et al. 2011). These data indicate that cerebellar nuclei neurons are capable of dramatically altering hippocampal activity and coding of spatial cues that may be

relevant for shaping EtOH consumption behavior in the context of craving. Therefore, determining which nuclei, neurons, and synapses are involved in this pathway may provide further insight into the role of the cerebellum in AUDs.

Finally, when considering the neurobiological underpinnings of EtOH consumption behaviors, it is essential to address the hypothalamus, a brain region responsible for a range of autonomic functions, regulating hormone secretion, and metabolic homeostasis. On its own, the hypothalamus has been shown to be important in mediating motivated behaviors to feed and seek out drugs of abuse or reward (Marchant et al. 2012). However, the hypothalamus also both sends and receives input to/from cerebellar nuclei neurons (Zhang et al. 2016; Zhu et al. 2006). In primate and rat, tract tracing suggests that neurons from all three cerebellar nuclei form projections that are broadly distributed across a number of posterior hypothalamic nuclei (Zhu et al. 2006; Zhang et al. 2016; Cavdar et al. 2001a, b; Haines et al. 1990), including the lateral hypothalamus which are known to be important in EtOH seeking behavior (Marchant et al. 2009; Dayas et al. 2008; Hamlin et al. 2007). However, outside of this tract tracing approach, little is known about the function of this pathway to influence behavior, or even the neurotransmitter systems and cell types in the hypothalamic nuclei that may be involved.

2.3 History of and Recent Controversies About Actions of EtOH Within the Cerebellum

Ultimately, fully understanding the actions of EtOH on human neurophysiology, and thus the etiology of and treatment for AUDs, requires determining the molecular and neural targets of EtOH. This sentiment is reflected in some of the first in vivo brain recording studies examining responses to systemic EtOH in various brain regions of the rat or rabbit (Klemm and Stevens 1974; Klemm et al. 1976). In this context, because of the clear adverse impact of EtOH on motor control and balance, many early in vivo studies of EtOH actions at a cellular level included or even focused on the cerebellum (Klemm and Stevens 1974; Klemm et al. 1976; Rogers et al. 1980; Deitrich et al. 1989), a presumed mediator of such EtOH-induced motor impairments. Such early studies determined that the cerebellum, along with the hippocampus and cerebral cortex, was one of the more EtOH-sensitive brain regions, generally responding to lower [EtOH] than other brain regions. In particular, most early in vivo studies used single unit recording from Purkinje cells (PCs), which are the sole output of the cerebellar cortex, and thus should reflect actions of EtOH anywhere in the cerebellar cortex. Such studies generally indicated that EtOH suppresses PC firing, although some studies also showed enhanced firing, potentially reflecting different doses of EtOH used and/or local direct versus secondary, upstream actions of EtOH (Deitrich et al. 1989). For example, Rogers et al. (1980) determined that systemic EtOH increased PC complex spike firing but simultaneously decreased their simple spike firing rates in an anesthetized preparation (Rogers et al. 1980). Given that PC complex spikes are synaptically driven by climbing fiber afferents from the inferior olive, whereas PCs fire simple spikes

spontaneously, the opposite actions within the same cell were interpreted as reflecting actions of EtOH in the inferior olive and direct actions on the PCs, respectively. Interestingly, such acute responses were absent in rats that had been chronically exposed to EtOH prior to recording, and climbing fiber driven complex spike frequency was reduced during withdrawal (Rogers et al. 1980). Thus, in addition to being highly sensitive to EtOH (acute intoxication), the cerebellum exhibits neural correlates of behavioral tolerance and dependence.

Parallel in vivo studies examining PC responses to local cerebellar application of EtOH, combined with studies of isolated slices of cerebellum confirmed that acute suppression of PC simple spikes by EtOH is indeed due to direct actions within the cerebellum (George and Chu 1984; Siggins and French 1979). Importantly, while such impacts could be due to direct actions of EtOH on PCs, they could also be influenced by the underlying granule cells, which synaptically modulate PC spike firing and also appear sensitive to the actions of EtOH in vivo. For example, low dose EtOH dramatically suppressed sensory-evoked granule cell spiking activity in cat upon systemic administration of low dose EtOH (0.3 g/kg = 15–20 mM EtOH in CSF) (Huang and Huang 2007). Therefore, EtOH-induced changes observed at the level of PCs may also reflect actions elsewhere in the cerebellar cortex.

Given the above summarized establishment of the cerebellum as a sensitive target of EtOH, consequent studies began to focus on two crucial issues that have yet to be fully resolved: (1) identifying the molecular targets that mediate EtOH impacts on cerebellar signal propagation and (2) determining if there are genetic differences in such cerebellum-specific actions that may relate to predilection for excessive alcohol consumption and addiction. In terms of the latter interest, a series of studies by Hoffer and colleagues determined that the degree of EtOH-induced spike suppression in PCs correlated with genetic variation in sensitivity to the soporific effects of EtOH in vivo (Basile et al. 1983; Palmer et al. 1982, 1985; Sorensen et al. 1980, 1981). In particular, PCs in mouse lines bred to be sensitive (long sleep mice; LS mice) to the soporific effects of EtOH (i.e., duration of sleep, as assessed by a loss of the righting reflex) were significantly more sensitive to EtOH-induced suppression of spiking than PCs in mice bred to be insensitive to the soporific effects of EtOH (short sleep mice; SS mice). Such differences are somewhat specific to the cerebellum, as parallel recordings of similarly sensitive hippocampal neurons did not show any differences in sensitivity between LS and SS mice (Sorensen et al. 1981). Importantly, the observed cerebellar differences persisted in acutely isolated slices of cerebellum and when the cerebelli from LS/SS mice were transplanted into the ocular space of the opposite line of mice, collectively confirming that such genetic differences were inherent properties of the cerebellum (Basile et al. 1983; Palmer et al. 1982, 1985). Finally, when LS and SS mice were chronically exposed to EtOH in vivo, they both developed behavioral tolerance to the ataxic and soporific effects of EtOH, which was mirrored by the development of tolerance at the level of PC spiking, even in slices. This suggests again that crucial aspects of AUD (acute sensitivity and tolerance) are exhibited by the cerebellum at both the behavioral and cellular level (Palmer et al. 1985).

Interest in the cerebellum as a primary mediator of EtOH intoxication was further stimulated by the contemporarily developing appreciation that $GABA_ARs$ were key mediators of EtOH intoxication (Allan et al. 1987; Harris et al. 1988; Palmer et al. 1988; Allan and Harris 1987), and that the newly developed $GABA_AR$ inverse agonist, Ro 15-4513, the so-called alcohol antagonist, had a high affinity for binding to the α6 subunit of $GABA_ARs$ that are fairly exclusively expressed on cerebellar granule cells (Fig. 1b) (Luddens et al. 1990). In particular, studies of EtOH actions on $GABA_ARs$ using membrane microsacs isolated from cerebellum determined that EtOH enhanced $GABA_AR$-stimulated Cl^- flux in LS mice, but not in SS mice (Allan and Harris 1986; Allan et al. 1987, 1988). And, similar to the cerebellar specificity that was observed with in vivo neuronal recordings, there were no differences in EtOH modulation of $GABA_AR$-stimulated Cl^- flux in membrane microsacs derived from the hippocampus of LS and SS mice. Lastly, EtOH suppression of Purkinje cell firing and enhancement of microsac Cl^- flux was significantly reduced by Ro 15-4513 (Harris et al. 1988; Palmer et al. 1988). Collectively, the data suggest that genetic differences in behavioral sensitivity to EtOH intoxication are mediated in part by genetic differences in the sensitivity of the cerebellar $GABA_AR$ system to EtOH.

Despite the genetic relationship between behavioral intoxication (as assessed by sleep time) and cerebellar sensitivity to EtOH, relating such differences to actual genetic variation in EtOH consumption has been more complicated. In particular, early studies of EtOH consumption by LS and SS mice determined that SS mice consumed more EtOH than LS mice when the consumption options were sweetened EtOH versus tap water (Church et al. 1979). Importantly, when given the choice between sweetened EtOH and sweetened tap water, although SS mice still consumed more EtOH than LS mice, they both consumed significantly less EtOH. Together, these outcomes suggest that at least part of the limit on EtOH consumption was driven by aversive aspects of EtOH which can be overridden to an extent by sweetening. While these outcomes support the broad idea that genetic sensitivity to aversive aspects of EtOH, particularly those mediated by the cerebellum (reflected in this case by loss of righting reflex), is a deterrent to abusive EtOH consumption, parallel operant studies determined that only LS mice exhibited positive reinforcement by EtOH, and thus worked for pharmacologically active levels of EtOH (Elmer et al. 1990). Subsequent studies with a variety of inbred or selected lines have revealed similar discrepancies, with levels of EtOH consumption being greater in either higher or lower sensitivity rodent genotypes, or not being correlated at all (Kakihana et al. 1966; Malila 1978; Spuhler and Deitrich 1984; Tabakoff and Kiianmaa 1982; Riley et al. 1977; Erwin et al. 1980; Millard 1983; Daoust et al. 1987). Thus, while there is frequent genetic covariation between EtOH consumption and "sensitivity" phenotype, the relationship is not always negative, and the two behavioral phenotypes are, although frequently genetically linked, separable.

In this context, it is important to emphasize that few, if any rodent genotypes will voluntarily consume enough EtOH to induce sleep, and the high [EtOH] required to induce sleep will obviously affect multiple molecular and neural targets that may not influence early, nondependent voluntary consumption, which complicates

interpretation of genetic risk for excessive EtOH consumption (Bell et al. 2001). Relatedly, although sleep duration and other measures of acute intoxication duration may reflect initial sensitivity, their duration is also affected by the rate of development of acute functional tolerance, which is a separable, genetically determined factor that presumably also influences the overall subjective initial "reaction" to EtOH (Crabbe et al. 2006a; Ponomarev and Crabbe 2002; Gallaher et al. 1996; Fritz et al. 2012). Further complicating interpreting the role of such genetic sensitivity in risk for abusive EtOH consumption, it is also possible that EtOH-induced sedation may be aversive to some genotypes and positive to others.

Collectively, the complications described above form part of the following rationale for focusing on responses to low [EtOH]. First, responses to low [EtOH] and genetic differences in such responses are key to determining the nature of initial reactions to EtOH in most nonhuman models and early non-abusive EtOH consumption by humans that may ultimately determine predilection to AUD. Second, the smaller number of molecular/neural targets of low [EtOH] will be more tractable and thus relatable to specific behavioral endophenotypes that influence development of AUD.

In this broad context, while most early in vivo studies of cerebellar responses to EtOH found PCs to be highly sensitive, parallel slice studies with synaptic transmission blocked found that the concentration of EtOH required to suppress PC firing directly were higher [30–100 mM (Basile et al. 1983)] than those required to induce motor incoordination (~10 mM). This discrepancy, combined with early evidence that low [EtOH] [10–15 mM (Allan and Harris 1986; Allan et al. 1988)] could enhance $GABA_AR$-mediated Cl^- flux in cerebellar-derived membrane microsacs prompted a shift in focus to other cellular targets in the cerebellar cortex that might underlie the higher in vivo sensitivity of the cerebellum to low [EtOH] (Carta et al. 2004; Freund et al. 1993). In particular, an early in vivo recording study determined that EtOH increased action potential firing of inhibitory Golgi cells that provide lateral and feedback inhibition to granule cells (Freund et al. 1993). Subsequently, a slice study confirmed that concentrations of EtOH as low as 10 mM increased Golgi cell spontaneous firing frequency, which increased GABA release onto granule cells, manifesting as increased phasic IPSC frequency and associated increase in magnitude of granule cell tonic $GABA_AR$ current (Carta et al. 2004).

While the initial slice study by Valenzuela and colleagues indicated that EtOH increased inhibition of granule cells by increasing Golgi cell firing (because EtOH did not affect granule cell inhibition in the presence of the sodium channel blocker tetrodotoxin) (Carta et al. 2004), a subsequent study by Olsen and colleagues argued that much of the enhancement of the granule cell tonic $GABA_AR$ current was mediated by direct actions of EtOH on the α6-δ-subunit containing extrasynaptic $GABA_ARs$ that generate granule cell tonic $GABA_AR$ currents (Hanchar et al. 2005). Moreover, these authors argued that a single point mutation in the α6 subunit conferred increased sensitivity of the granule cell tonic $GABA_AR$ current to EtOH, and that the increased sensitivity resulted in increased behavioral sensitivity to the motor impairing effects of EtOH. This study, along with parallel studies of cloned $GABA_ARs$ (Wallner et al. 2003) heralded an exciting moment in EtOH research in

which the researchers concluded that the δ subunit, and its typical pairing with either the α4 (hippocampus and thalamus) or α6 (cerebellum) $GABA_AR$ subunits was the elusive "one glass of wine receptor" that mediated the well-established behavioral sensitivity to low [EtOH] (Olsen et al. 2007). While the authors also observed EtOH-induced increased release of GABA from Golgi cells, they have concluded that most of that increase is driven by EtOH directly enhancing δ-subunit-containing $GABA_ARs$ on the axons of granule cells, which because they are excitatory (Pugh and Jahr 2011, 2013), actually enhance excitatory synaptic drive of Golgi cell-mediated feedback inhibition of granule cells (Santhakumar et al. 2013). Unfortunately, such direct actions on $GABA_ARs$ of low [EtOH] have not been observed by most other researchers, ourselves included, either in situ or in cloned $GABA_ARs$, and the cause of such discrepancies remains unclear [for detailed discussions of this ongoing controversy see Santhakumar et al. (2007); Korpi et al. (2007); Borghese and Harris (2007); Botta et al. (2007a, b); Olsen et al. (2007); Valenzuela and Jotty (2015)].

While we predict that eventually the discrepancy will be discovered to stem from some subtle difference across labs in tissue health and/or intracellular milieu (such as intracellular $[Ca^{2+}]$, [NO], or phosphorylation status), we will provide a brief description of why we conclude that direct enhancement is not likely to be a dominant mediator of EtOH actions in the cerebellum or its role in AUD. First, similar to Valenzuela's findings, in a range of mammalian species, including non-human primates, we have been unable to see any direct enhancement of granule cell tonic $GABA_AR$ currents by even high [EtOH] (9–105 mM) (Kaplan et al. 2013, 2016a; Mohr et al. 2013). Importantly, while concluding that there is direct enhancement in situ is dependent on ensuring that all possible sources of increased GABA are prevented, the converse is not true: a lack of enhancement cannot be explained by inadequate block of a potential source of GABA. Thus, we find the complete absence of EtOH enhancement of granule cell tonic $GABA_AR$ currents in the presence of tetrodotoxin in B6 and D2 mice, SD rats, prairie voles, and nonhuman primates to be compelling evidence that EtOH does not directly enhance granule cell tonic $GABA_AR$ currents (Kaplan et al. 2013, 2016a; Mohr et al. 2013). Similarly, we also reported that in a subset of granule cells in B6 and D2 mice, and nonhuman primates, EtOH did not affect the tonic $GABA_AR$ current even without blocking action potentials, which was associated with a lack of increase in sIPSC frequency, as well as low nNOS expression, which we have determined is a key mediator of EtOH excitation of Golgi cells (Kaplan et al. 2013). Importantly, the lack of enhancement in our hands cannot be due to a ceiling effect, because in all mammalian species we have tested, bath application of THIP [at concentrations that are absolutely selective for δ-subunit-containing $GABA_ARs$ (Meera et al. 2011)] increases the magnitude of the tonic $GABA_AR$ current (Kaplan et al. 2013, 2016a; Mohr et al. 2013). Finally, as will be discussed in greater detail below, we recently discovered that EtOH actually directly suppresses tonic $GABA_AR$ currents in high EtOH consuming genotypes (B6 mice and prairie voles), and this suppression persists when granule cells are physically removed from the slice, thus precluding possible actions of EtOH-induced altered GABA release (Kaplan et al. 2013, 2016a).

Similarly compelling, it was recently shown that EtOH increases tonic $GABA_AR$ currents in pre-weanling SD rats that do not yet express the δ-subunit of the $GABA_AR$, with the cause of the enhancement again being increased GABA release (Diaz and Valenzuela 2016). This is particularly important because although germline deletion of the δ-subunit reduces responses to EtOH (Santhakumar et al. 2013), it is well known that germline deletion of $GABA_ARs$ causes a variety of homeostatic adaptations in the cerebellum which could indirectly alter responses to EtOH (Valenzuela and Jotty 2015; Brickley et al. 2001).

Finally, regarding the proposed role of excitatory, axonal δ-containing $GABA_ARs$ in mediating EtOH-induced increased GABA release from Golgi cells (Santhakumar et al. 2013), we have done extensive immunocytochemistry for the δ-subunit in the cerebellar cortex, and we have seen no evidence for it being expressed in the molecular layer where the granule cell axons reside, despite robust expression within the granule cell layer (Kaplan et al. 2013; Mohr et al. 2013). Moreover, we were able to significantly reduce EtOH-increased Golgi cell firing and consequent GABA release onto granule cells by blocking nNOS, without blocking the glutamate receptors that were hypothesized to drive Golgi cell excitation (Kaplan et al. 2013). In this regard it is important to note that it is possible that blocking glutamate receptors will reduce basal Ca^{2+} influx and thus reduce nNOS activity, thereby circumventing EtOH from exciting Golgi cells via nNOS block, independent of EtOH-induced changes in glutamatergic synaptic transmission.

In summary, although there are clearly still some specific cross-lab conflicting results, many of the apparent conflicts can be explained by alternative interpretations of complex network and molecular interactions. Regardless, since the genotypic differences we will consider below were discovered in our lab using identical techniques and solutions, any differences in EtOH actions and associated impacts on EtOH consumption phenotypes should be specific to true genetic variation.

2.4 Genetic Variation in Cerebellar Cortical $GABA_AR$ Signaling Responses to EtOH Influences EtOH Consumption Phenotype

As discussed above, the cerebellum is exquisitely sensitive to EtOH, with concentrations as low as 10 mM significantly altering cerebellar neural signaling (Kaplan et al. 2013, 2016b; Welsh et al. 2011; He et al. 2013; Richardson and Rossi 2017) and, consequently, known cerebellar-dependent behaviors (Gallaher et al. 1996). Importantly in the context of genetic predilection to AUD, over the last several years we have discovered that the response of key cerebellar processes to low [EtOH] varies across mammalian genotypes in a manner that correlates with, and appears to influence EtOH consumption phenotype (Mohr et al. 2013; Kaplan et al. 2013, 2016a, b; Richardson and Rossi 2017). While there are many molecular targets of EtOH in the cerebellum, which collectively affect almost all cells and synapses within the cerebellar cortical circuit (Dar 2015; He et al. 2013; Kaplan et al. 2013; Richardson and Rossi 2017; Welsh et al. 2011; Valenzuela and Jotty 2015), only a few targets have been identified that are sensitive to 10 mM EtOH, including

T-type Ca^{2+} channels in the Inferior Olivary neurons that provide climbing fiber inputs to Purkinje cells (Welsh et al. 2011), the NMDA receptors in the climbing fiber to Purkinje cell synapse (He et al. 2013; Zamudio-Bulcock et al. 2018), and the $GABA_AR$ synapse from Golgi cells to granule cells, which has both Golgi cell and postsynaptic targets (Kaplan et al. 2013). Indeed, we explicitly determined that 10 mM EtOH does not have any detectable impact on any of the other cells and synapses in the mouse cerebellar cortex (Kaplan et al. 2016b). And, while the two climbing fiber targets have been implicated respectively in EtOH withdrawal-induced tremors (Olivary neuron T-type Ca^{2+} channels) and cerebellar learning (Purkinje cell NMDA receptors), both of which may influence predilection to AUD, to our knowledge, only the Golgi cell to granule cell synapse is known to vary in its response to low [EtOH] in a manner that correlates with EtOH consumption phenotype across mammalian genotypes. Thus, for the remainder of this chapter, we will focus on the Golgi cell to granule cell synapse.

As introduced above, in low EtOH consuming Sprague Dawley rats (SDRs), low [EtOH] (starting at ~10 mM) enhances Golgi cell inhibition of granule cells (Carta et al. 2004). The enhancement manifests as both an increase in the frequency of sIPSCs and an increase in the magnitude of the tonic $GABA_AR$ current (Figs. 1d and 3a), which in granule cells is mediated by extrasynaptic α6 and δ subunit-containing $GABA_ARs$ (Hamann et al. 2002). Despite the controversy described above (Hanchar et al. 2005; Santhakumar et al. 2007; Korpi et al. 2007; Borghese and Harris 2007; Botta et al. 2007a, b), we believe that the preponderance of data indicates that the primary mechanism is via EtOH-induced increased action potential firing by Golgi cells, which accounts for the increase in both sIPSC frequency and in tonic $GABA_AR$ current magnitude, due to elevated extracellular GABA. These fundamental observations have been replicated by several labs, including those that also see direct enhancement of $GABA_ARs$ (Hanchar et al. 2005; Kaplan et al. 2013), and are part of the cornerstone of two widely accepted concepts: (1) a main target of recreational [EtOH] is the $GABA_AR$ system generally, and (2) EtOH-induced motor impairment is due to enhancement of Golgi cell inhibition of granule cells by EtOH (Hanchar et al. 2005; Dar 2015).

The notably high sensitivity of the Golgi cell to granule cell $GABA_AR$ system to EtOH, combined with the clear role of this response in mediating at least one behavioral response to EtOH (motor impairment) makes it a potential mediator of genetic differences in response to low [EtOH] that influences initial subjective reactions to consumption of EtOH, and thus predilection for developing an AUD. In this context, we decided to determine if the action of EtOH on the Golgi to granule cell $GABA_AR$ system varied across rodent genotypes with differing EtOH consumption phenotypes (Fig. 3a–d) (Kaplan et al. 2013). Indeed, in our initial study we found that in stark contrast to the enhancement of inhibition induced by EtOH in low EtOH consuming SDRs, EtOH actually suppressed the magnitude of the granule cell tonic $GABA_AR$ current in the prototypical high EtOH consuming C57BL/6J mice (B6; Fig. 3b–d). This divergence is not simply a species difference, because EtOH enhanced sIPSC frequency and tonic $GABA_AR$ current magnitude in the low EtOH consuming DBA/2J mouse (D2; Fig. 3a–c). Parallel studies in high EtOH consuming

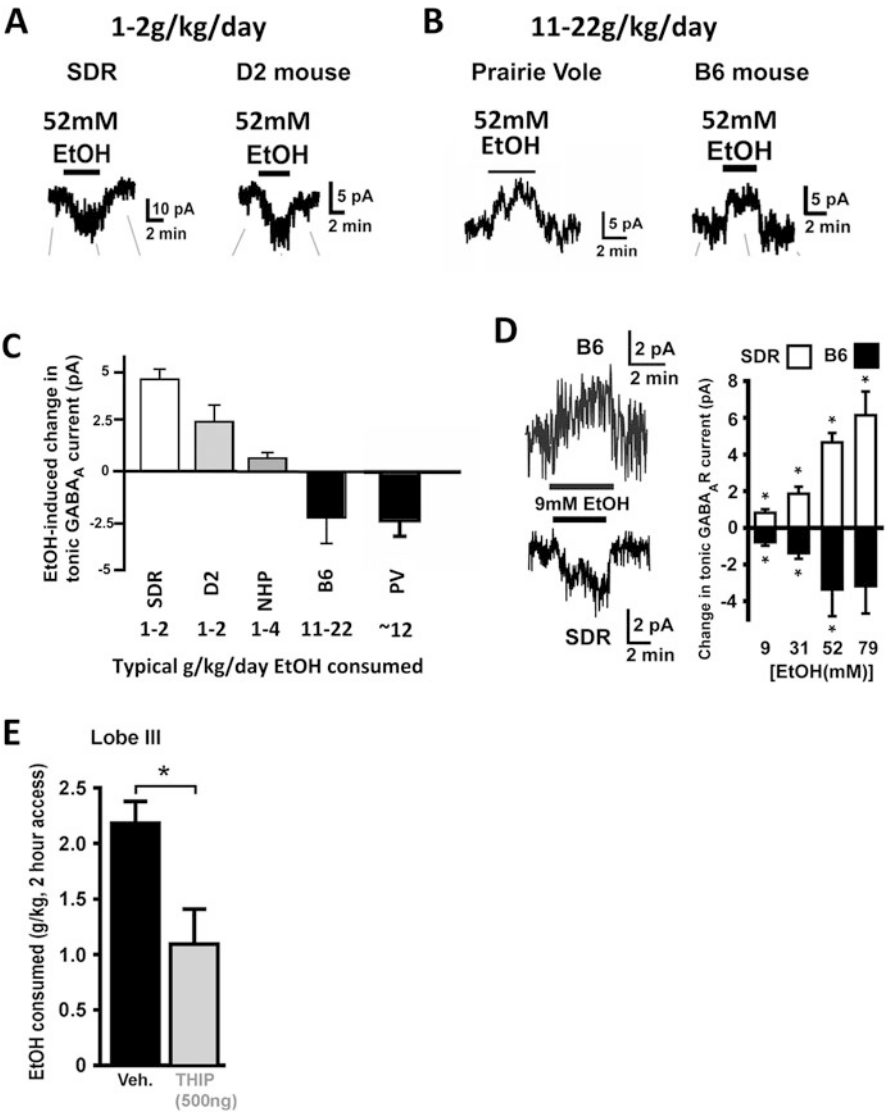

Fig. 3 Response of granule cell tonic $GABA_AR$ current to EtOH varies in parallel with and influences EtOH consumption phenotype. (**a**, **b**) Example recordings showing that EtOH (52 mM) enhances the tonic $GABA_AR$ current in low EtOH consuming rodent genotypes (SDRs and D2 mice; **a**), but suppresses the tonic $GABA_AR$ current in high EtOH consuming rodent genotypes (Prairie Voles and B6 mice; **b**). (**c**) Plot of mean EtOH-induced change in magnitude of granule cell tonic $GABA_AR$ current across mammalian genotypes with divergent EtOH consumption phenotypes. **Note**, EtOH consumption values are rough estimates of average amount consumed across a 24 h period for each mammalian genotype, without consideration for consumption pattern across the day. (**d**) Example recordings and bar chart of mean responses to varying doses of EtOH in SDRs and B6 mice showing that opposite action of EtOH is preserved at low to high [EtOH]. (**e**) Bar chart depicts the mean amount of EtOH consumed by B6 mice during a 2 h 2 bottle choice

prairie voles (PVs) and variable EtOH consuming nonhuman primates (NHPs) revealed a general pattern in which the impact of EtOH on granule cell tonic $GABA_AR$ currents varies in polarity and magnitude in parallel with the EtOH consumption phenotype of the mammal, with high and low EtOH consumption associated with suppression and enhancement of tonic $GABA_AR$ currents respectively (Fig. 3b, c). Crucially, this relationship persists across the dose–response range of EtOH concentrations tested, including 9 mM (Fig. 3d). To test whether this striking correlation actually played a role in EtOH consumption, we attempted to counteract the ability of EtOH to suppress the tonic $GABA_AR$ current in B6 mice in vivo. To do this, the $GABA_AR$ agonist THIP, which has an order of magnitude higher affinity for δ-subunit containing $GABA_ARs$, was focally injected into the cerebellum of B6 mice in vivo. This would effectively increase granule cell tonic $GABA_AR$ currents similar to what EtOH does in low EtOH consuming genotypes (Kaplan et al. 2013). Such injections effectively reduced EtOH consumption by B6 mice (Fig. 3e), without affecting water consumption or overall locomotion (Kaplan et al. 2016b). While more selective manipulations of the tonic $GABA_AR$ current will need to be tested, and ideally it should be determined if EtOH consumption can be increased or decreased based on the direction of manipulation, these studies suggest that either EtOH-induced suppression of granule cell tonic $GABA_AR$ currents promotes EtOH consumption or EtOH-induced enhancement of granule cell tonic $GABA_AR$ currents deters consumption, or both.

It is worth noting that although the cerebellar neurological differences that we discovered clearly correlate with and influence EtOH consumption (Fig. 3c, e) (Kaplan et al. 2016b), they are not the only factors that influence EtOH consumption. Of particular relevance, it is well established that a large factor in deterring EtOH consumption by D2 mice is their aversion to the taste of EtOH, and an apparent insensitivity to glucose, which undermines using it as a means to override taste aversion (Grahame and Cunningham 1997; McCool and Chappell 2012, 2014; Fidler et al. 2011). Thus, when taste is bypassed by intravenous or gastric cannulation, D2 mice will self-administer significant quantities of EtOH (Grahame and Cunningham 1997; Fidler et al. 2011). Similarly, D2 mice will also voluntarily orally consume EtOH if its taste is masked with monosodium glutamate (McCool and Chappell 2012, 2014). However, even with such aversive taste circumventions, D2 mice still consume significantly less EtOH than B6 mice, suggesting that aversive post-absorptive ethanol effects probably contribute to avoidance of oral consumption of ethanol by D2 mice (McCool and Chappell 2012, 2014; Fidler et al. 2011). In this regard, when normalized to the basal magnitude of tonic $GABA_AR$ current, the percent change induced by EtOH in D2 mice falls roughly between the percent change observed in B6 mice and SD rats, being significantly different from both (Kaplan et al. 2016a). This is interesting because unlike D2 mice, SD rats find

Fig. 3 (continued) (water and 10% EtOH) session, under control conditions and after a local injection of the $GABA_AR$ agonist, THIP, into lobe 3 of the cerebellum. Adapted with permission from Kaplan et al. (2013, 2016a, b)

EtOH aversive even when taste is bypassed (Fidler et al. 2004). Thus, EtOH actions on granule cell tonic $GABA_AR$s may contribute to deterrence of EtOH consumption in D2 mice relative to B6 mice, but not as strongly as it does in SD rats.

In these studies we also determined the molecular mechanisms that determine the overall response polarity. Specifically, we found that enhancement of tonic $GABA_AR$ currents is mediated by EtOH inhibition of neuronal nitric oxide synthase (nNOS), which excited Golgi cells enough to increase their action potential firing (Kaplan et al. 2013), possibly via NO actions on the Na^+/K^+-ATPase and/or K^+ channels (Botta et al. 2010, 2012; Valenzuela and Jotty 2015). Genetic control of this process appears to be implemented by expression of nNOS, with low levels of expression in high EtOH consuming B6 mice and PVs, high levels of expression in low EtOH consuming SDRs and D2 mice, and intermediate and variable levels of expression across individual NHPs which also show variable levels of EtOH consumption (Kaplan et al. 2013, 2016a; Mohr et al. 2013). Conversely, the suppression of granule cell tonic $GABA_AR$ currents observed in B6 mice and PVs is mediated solely by postsynaptic actions on the $GABA_AR$s, as evidenced by the fact that suppression was observed in acutely isolated granule cells that had their $GABA_AR$s activated by exogenous GABA to circumvent any possible action of EtOH on endogenous GABA release (Kaplan et al. 2013). And, we determined that the ability of EtOH to postsynaptically suppress granule cell tonic $GABA_AR$ currents is genetically determined by the level of postsynaptic PKC activity. In particular, PKC activity appears to prevent EtOH from suppressing granule cell tonic $GABA_AR$ currents. Thus, activating PKC in B6 mouse granule cells eliminated EtOH suppression of tonic $GABA_AR$s, whereas blocking PKC in SDR granule cells enabled EtOH to suppress their tonic $GABA_AR$ currents (Kaplan et al. 2013).

Collectively, our data indicate that there are two genetically controlled molecular switches (nNOS expression and postsynaptic PKC activity), and that the balance of the two processes dictates the polarity and magnitude of the effect of EtOH on granule cell tonic $GABA_AR$ currents. High postsynaptic PKC activity and high nNOS expression result in EtOH enhancing granule cell tonic $GABA_AR$ currents, and low postsynaptic PKC activity and low nNOS expression result in EtOH suppressing tonic $GABA_AR$ currents. Importantly, the resultant direction of EtOH effects correlates with and influences EtOH consumption phenotype, wherein suppression and enhancement are associated with high and low EtOH consumption, respectively.

2.5 Is There Potential for Manipulating the Cerebellum to Deter EtOH Consumption with Fewer Side Effects?

Based on our recent studies, our overall contention is that EtOH suppression of granule cell tonic $GABA_AR$ currents promotes EtOH consumption in high EtOH consuming genotypes, whereas EtOH enhancement of such currents deters EtOH consumption in low EtOH consuming genotypes (Fig. 3a–d). Accordingly, we propose that pharmacological agents that selectively enhance granule cell tonic

$GABA_AR$ currents, or can emulate the overall outcome of such enhancement on PC output (presumed to be the final mediator of any cerebellar behavior), should reduce EtOH consumption. And, given that granule cell tonic $GABA_AR$ currents are mediated by α6-subunit containing $GABA_ARs$, which are almost exclusively expressed by granule cells (Fig. 1c), any such pharmacotherapy might be achieved with fewer side effects than more broadly acting $GABA_AR$ modulators.

As discussed above, Ro 15-4513 strongly binds to the α6 containing $GABA_ARs$ that generate tonic $GABA_AR$ currents in granule cells (Luddens et al. 1990). Although Ro 15-4513 is generally considered to be an inverse agonist of $GABA_ARs$, it has been shown to enhance currents generated by α6 containing $GABA_ARs$ (Knoflach et al. 1996). In partial support of our broad contention, in at least one study of alcohol preferring (AA) rats, Ro 15-4513 did in fact decrease EtOH consumption (Wegelius et al. 1994). However, Ro 15-4513 is clearly not selective for the α6 subunit or the cerebellum. So, to more directly test the hypothesis, we have shown that a local injection of THIP into the cerebellum effectively reduces EtOH consumption by B6 mice without affecting water consumption or general locomotion (Fig. 3e) (Kaplan et al. 2016b). While this confirms the potential capacity of targeting the cerebellar $GABA_AR$ system to treat AUDs, as discussed above, it is unlikely that THIP will be a suitable therapeutic in the human clinic, due amongst other things to the widespread action of THIP and associated adverse side effects.

However, having determined that regulating granule cell excitability, and presumably therefore Purkinje cell output, can effectively reduce EtOH consumption without obvious adverse effects, it is reasonable to speculate that any drug that could specifically target cerebellar granule cell excitability or Purkinje cell output might be an effective treatment option. Accordingly, we will end this chapter by highlighting the fact that the cerebellum is an unusual brain structure in that there are a variety of subunits/subtypes of common receptor/channel/transporter families that are exclusively expressed in the cerebellum, many only on cerebellar granule cells. Importantly, many of these cerebellar, or even granule cell specific subunits are known to be key players in regulating signal transmission through the cerebellar cortex, including the α6 subunit of the $GABA_AR$ family (Fig. 1c) (Hamann et al. 2002), the EAAT4 subtype of the plasma membrane glutamate transporter (Kaplan et al. 2016b; Wadiche and Jahr 2005; Welsh et al. 2002), and the NR2C subunit of the NMDA receptor family (Cathala et al. 2000, 2003; Ebralidze et al. 1996). Thus, there are likely many potential targets for modulating cerebellar responses to EtOH that may not affect the rest of the brain. Indeed, $GABA_AR$-positive allosteric modulators have already been developed that show orders of magnitude selective affinity for α6 containing $GABA_ARs$ (Varagic et al. 2013). Given that enhancement of granule cell tonic $GABA_AR$ currents reduces EtOH consumption (Fig. 3e) (Kaplan et al. 2016b), we would predict that such ligands could reduce EtOH consumption, and given their selectivity for cerebellar granule cells, may have fewer side effects than drugs that target more widely expressed $GABA_AR$ subunits.

3 Summary

It is clear that the $GABA_AR$ system plays multiple important roles in mediating acute and chronic responses to EtOH and almost certainly plays a role in the development and maintenance of AUD. However, such information has not translated to effective treatment for AUD. Contributing factors to this failure likely include the inadequate understanding of: (1) the molecular/neural targets of low [EtOH] (~10 mM), (2) the neural systems that mediate initial reactions to low [EtOH], and (3) pharmaceuticals that can selectively target relevant neural systems in a manner that is not plagued by side effects.

Recently, our understanding of non-motor roles of the cerebellum has evolved dramatically, and we now know that the cerebellar $GABA_AR$ system is highly sensitive to EtOH, responding to [EtOH] as low as 10 mM. Moreover, variability in the polarity and magnitude of the response of cerebellar $GABA_ARs$ to EtOH correlates with and actually affects EtOH consumption phenotype across various mammalian genotypes. This suggests that the cerebellum generally, and the cerebellar $GABA_AR$ system specifically may be promising targets for AUD pharmacotherapy. Importantly, the cerebellum and its $GABA_AR$ system are unique in their pattern of expression of atypical subunits/subtypes of $GABA_ARs$ and other transmitter systems that could be targeted, potentially without producing typical adverse side effects of drugs that affect more widely distributed targets.

Acknowledgments This work was supported by National Institute on Alcohol Abuse and Alcoholism Grants R01AA-012439 and R01AA-026078, and Washington State University Alcohol and Drug Abuse Research Program (ADARP) grant to D.J.R., and by an ADARP postdoctoral grant to B.D.R.

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Do Alcohol-Related AMPA-Type Glutamate Receptor Adaptations Promote Intake?

F. Woodward Hopf and Regina A. Mangieri

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Abstract

Ionotropic glutamate receptors (AMPA, NMDA, and kainate receptors) play a central role in excitatory glutamatergic signaling throughout the brain. As a result, functional changes, especially long-lasting forms of plasticity, have the potential to profoundly alter neuronal function and the expression of adaptive and pathological behaviors. Thus, alcohol-related adaptations in ionotropic glutamate

F. Woodward Hopf (✉)
Alcohol Center for Translational Genetics, Department of Neurology, University of California at San Francisco, San Francisco, CA, USA
e-mail: Frederic.Hopf@ucsf.edu

R. A. Mangieri
Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_105

receptors are of great interest, since they could promote excessive alcohol consumption, even after long-term abstinence. Alcohol- and drug-related adaptations in NMDARs have been recently reviewed, while less is known about kainate receptor adaptations. Thus, we focus here on functional changes in AMPARs, tetramers composed of GluA1–4 subunits. Long-lasting increases or decreases in AMPAR function, the so-called long-term potentiation or depression, have widely been considered to contribute to normal and pathological memory states. In addition, a great deal has been learned about the acute regulation of AMPARs by signaling pathways, scaffolding and auxiliary proteins, intracellular trafficking, and other mechanisms. One important common adaptation is a shift in AMPAR subunit composition from GluA2-containing, calcium-impermeable AMPARs (CIARs) to GluA2-lacking, calcium-permeable AMPARs (CPARs), which is observed under a broad range of conditions including intoxicant exposure or intake, stress, novelty, food deprivation, and ischemia. This shift has the potential to facilitate AMPAR currents, since CPARs have much greater single-channel currents than CIARs, as well as faster AMPAR activation kinetics (although with faster inactivation) and calcium-related activity. Many tools have been developed to interrogate particular aspects of AMPAR signaling, including compounds that selectively inhibit CPARs, raising exciting translational possibilities. In addition, recent studies have used transgenic animals and/or optogenetics to identify AMPAR adaptations in particular cell types and glutamatergic projections, which will provide critical information about the specific circuits that CPARs act within. Also, less is known about the specific nature of alcohol-related AMPAR adaptations, and thus we use other examples that illustrate more fully how particular AMPAR changes might influence intoxicant-related behavior. Thus, by identifying alcohol-related AMPAR adaptations, the specific molecular events that underlie them, and the cells and projections in which they occur, we hope to better inform the development of new therapeutic interventions for addiction.

Keywords
AMPA · Electrophysiology · GluA1 · GluA2 · Glutamate · Plasticity

1 General Introduction to AMPARs and Plasticity Related to Intoxicant Exposure

A great deal has been learned and written about AMPAR regulation and function (see Malinow and Malenka 2002; Collingridge and Isaac 2003; Fukata et al. 2005; Chen et al. 2010; Traynelis et al. 2010; Mao et al. 2011; Bats et al. 2013; Henley and Wilkinson 2013; Hanley 2014; Wang et al. 2014). Thus, our purpose here is to present a simplified version of the better studied aspects of AMPAR regulation, in order to understand the possible cellular and behavioral impact of AMPAR adaptations that are observed after exposure to alcohol or other intoxicants.

AMPARs are one of the three types of ionotropic, excitatory glutamate-activated receptors, in addition to NMDA receptors and kainic acid receptors. Importantly, glutamate receptor neuro-adaptations, especially an increase in function, have the potential to strongly drive maladaptive behavior including excessive alcohol drinking. We focus here on AMPARs, since NMDA receptor adaptations are addressed in several recent reviews (Hopf 2017; Morisot and Ron 2017) and kainic acid receptors are less understood but can contribute to alcohol behavior (Crowder et al. 2002; Acosta et al. 2012; Bach et al. 2015).

AMPARs are widely distributed through the brain and are typically the major glutamate receptor that mediates excitatory glutamatergic signaling at the hyperpolarized physiological resting potential in neurons (but see Hopf 2017). AMPARs are homo- and hetero-tetramers composed of GluA1–4 subunits. As addressed more fully below, a considerable amount is known about a number of specific molecular steps that control AMPAR activity, including phosphorylation events, scaffolding and auxiliary proteins, and changes in subunit composition. While some common themes do emerge (Fig. 1), there are also important nuances and some mixed findings (e.g., Fukata et al. 2005; Engblom et al. 2008; Henley and Wilkinson 2013). In part, the presence of multiple, redundant, and conflicting pathways for regulating AMPAR function makes sense for a molecule so fundamental to excitatory neuronal interactions. Thus, we will use both alcohol-related and other examples to better illustrate how a given regulatory step or adaptation can impact AMPAR function and behavioral expression.

When trying to uncover AMPAR adaptations and their behavioral impact, there are several general key points. First, AMPAR function can be precisely determined using ex vivo electrophysiology in live neurons in a brain slice. These methods were used to first identify activity-dependent, long-lasting increases in AMPAR function that are proposed to contribute to memory, the so-called long-term potentiation (LTP) (Malinow and Malenka 2002). Thus, the section "*Ex vivo* electrophysiology

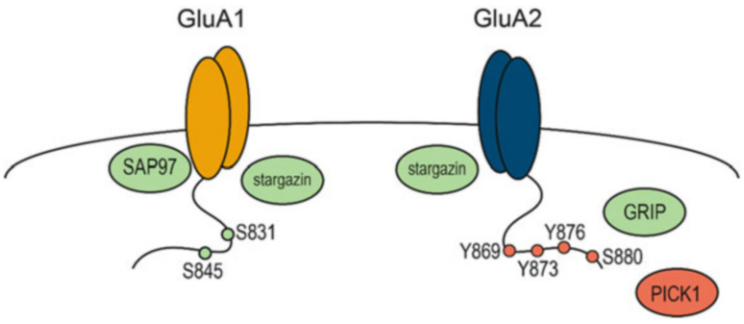

Fig. 1 Cartoon indicating several widely studied interactions for GluA1 and GluA2 AMPAR subunits, including critical phosphorylation sites and interacting molecules that alter both AMPAR localization and function. Green indicates molecules or phosphorylation sites that have been associated with retaining AMPARs at the membrane or enhancing AMPAR function, while red indicates signaling that can mediate internalization or intracellular retention of AMPARs

measures for AMPARs" below describes different experimental techniques that can be used to measure AMPAR function, and the particular strengths and limitations of each method. When functional measures agree, one can have greater confidence that a given adaptation has occurred. However, many different forms of AMPAR plasticity have been identified, and we believe it is critical in practice to perform a broad assessment of AMPAR function, including biochemical measures of AMPAR activity and localization within the cell, when trying to understand the importance of a particular AMPAR adaptation or signaling action. We also note that memory often involves new spine growth, and structural changes often occur during conditions of intoxicant enhancement of AMPAR activity (e.g., Passafaro et al. 2003; Beckley et al. 2016; Kalivas and Kalivas 2016).

Another general principle is the presence of calcium-permeable versus calcium impermeable AMPARs (CPAR or CIARs, respectively). In general, CPARs are enriched in GluA1 subunits and are GluA2-lacking (see Cull-Candy et al. 2006). Relative to CIARs, CPARs have nearly double the single-channel conductance and faster onset and offset kinetics. Thus, one-for-one exchange of GluA1s for GluA2s might be predicted to increase AMPAR currents. Calcium signaling through the calcium-permeability of CPARs also likely has important functional consequences, ranging from insertion of GluA2 subunits which stabilizes LTP (Liu and Cull-Candy 2000; Henley and Wilkinson 2013; Wu et al. 2017), to neurotoxicity during ischemia (Gerace et al. 2015). Also, CPARs are inhibited intracellularly by polyamines when the cell is at more depolarized potentials, which has provided a number of selective CPAR inhibitors that act at the polyamine site, including the widely used 1-naphthyl acetyl spermine (NASPM) and a range of selective toxins (Isaac et al. 2007).

In many brain regions, AMPAR function is primarily or exclusively mediated through CIARs (Conrad et al. 2008; Reimers et al. 2011; Ding et al. 2014), in agreement with significant protein levels of both GluA1 and GluA2 subunits (although some cells have basal CPAR activity, e.g., McGee et al. 2015). Importantly, alcohol exposure or intake leads to expression of CPARs in several brain regions (see below), which has the potential to promote excessive drinking. As discussed in detail below, an increase in CPARs, especially in the NAc, is observed after exposure to a number of behavioral conditions ranging from alcohol or cocaine exposure to stress or novelty. This long-term increase in CPARs may reflect their role in learning in memory, and reflect long-term storage of intoxicant-related approach memories. When tested, the increase in CPARs is critical for behavioral expression, for example, where NAc CPARs mediate cocaine relapse after protracted abstinence (e.g., Conrad et al. 2008). Overall, it is clear that CPAR induction represents a central form of insult-related adaptation that can strongly bias future behavior.

There are also likely to be intoxicant-related AMPAR adaptations that do not involve CPARs. An increase in both GluA1 and GluA2 subunit levels could elevate AMPAR activity without causing CPAR formation. In addition, stabilizing GluA2 surface expression and function (in a GluA2-K882A knockin mouse) increases AMPAR function without functional expression of CPARs, and also strongly enhances behavioral response to stressors and cocaine reinstatement and CPP (Briand et al. 2016; Ellis et al. 2017).

We discuss a number of examples of AMPAR regulation and adaptations for intoxicants other than alcohol, since these provide indications of other kinds of AMPAR signaling that could be observed, even if they have not yet been identified in relation to alcohol. However, it is also clear that different abused drugs can act through different circuits, e.g., psychostimulants versus opiates or alcohol (e.g., Marchant et al. 2015), and one might predict a priori that alcohol-related adaptations will be different from those with cocaine. Indeed, there are examples where psychostimulants produce different AMPAR changes relative to other drugs. It is equally important and interesting to understand which AMPAR adaptations diverge across intoxicants, and which AMPAR changes are common.

Finally, while AMPAR activity is clearly essential for a great deal of neural activity, and increased AMPAR function can promote intoxicant intake or seeking, it would be especially useful to understand whether intoxicant-related AMPAR adaptations occur in humans and are relevant for human alcohol addiction. This is challenging, as glutamate and related excitatory transmitters can be measured with MRS (Zahr et al. 2016). Cocaine can change NAc AMPARs in primates and humans (Hemby et al. 2005), but AMPARs will not be able to be directly studied until there is a PET ligand for the receptor (e.g., how raclopride is used to examine human dopamine-2-receptors). Nonetheless, several human studies have examined whether alcohol problems are related to gene variants across a cluster of AMPAR-related proteins. For example, Karpyak et al. (2012) found that gene variants in the NMDAR-dependent AMPAR trafficking pathway predict alcohol dependence. Also, gene variants in the mGluR-dependent AMPAR trafficking pathway are associated with greater problem alcohol drinking (Meyers et al. 2015). In addition to linking human drinking to AMPAR regulation, mice could be engineered to express to a specific gene variant linked to human drinking, which would allow better assessment of the physiological impact of the particular AMPAR-related genetic variant. Also, evidence for a relationship between AMPAR function in humans and alcohol addiction validates the possibility of using pharmacological agents that modulate AMPARs to decrease harmful drinking patterns (Holmes et al. 2013; Watterson and Olive 2013).

2 Ex Vivo Electrophysiology Measures to Measure AMPAR Function

Researchers often want to determine whether there is an increase or decrease in AMPAR function, perhaps after observing changes in AMPAR subunit levels with western blot. Ex vivo electrophysiology, where one records from a neuron in the live brain slice, provides powerful methods to directly assess glutamate receptor activity. It is simple to place two wires close together in the brain slice, and pass brief electrical current across them to stimulate glutamate release from terminals. Thus, it should be straightforward to detect changes in AMPAR function after alcohol or some other exposure. However, in practice, there can be slice-to-slice variability in currents generated this way, perhaps due to differences in geometry or survival of axons from different glutamate inputs. Thus, determining a change in the absolute magnitude of AMPARs is often the most difficult to address directly.

The AMPAR-to-NMDAR ratio is widely used to circumvent this problem. Measuring the ratio of AMPARs to NMDARs should control for possible differences in glutamate release across slices, since, e.g., lower release with electrical stimulation would reduce the magnitude of both AMPARs and NMDARs. In fact, NMDAR adaptations are observed (Hopf 2017; Morisot and Ron 2017), but longer-term functional changes in AMPARs are more common. Determining the AMPAR-NMDAR ratio is technically simple, where AMPARs are measured when holding a cell at −70 mV or +40 mV, then AMPARs are blocked pharmacological to measure NMDAR currents at +40 mV. In practice, this measure has been of immense value to identify AMPAR adaptations which may represent a form of LTP, and where elevated AMPAR function would strongly drive addiction-related behavior.

One clear caveat is that changes in NMDAR function will also alter the AMPAR-to-NMDAR ratio. Thus, one can vary the level of electrical stimulation in a brain slice and measure the amount of receptor activity at each level, called an input–output relationship. These more direct measures of pharmacologically isolated synaptic AMPAR or NMDAR currents tend to be more variable, relative to the AMPAR-to-NMDAR ratio, and require a larger sample size to accurately assess receptor function. Other groups have also used brief (10–30 s) bath application of AMPA or NMDA as another method to assess functional changes, which can be complicated by unnatural AMPAR desensitization or by activation of extra-synaptic receptors. Nonetheless, these measures can provide valuable confirmation when other evidence suggests an increase in AMPAR function.

Changes in AMPAR function can also be assessed through AMPAR currents generated by spontaneous release of glutamate-containing vesicles. These so-called miniature excitatory postsynaptic currents (mEPSCs) are recorded in the presence of sodium channel blockers (lidocaine or tetrodotoxin) to prevent action potential firing in presynaptic axons, which assures that postsynaptic mEPSCs represent activation by a single glutamate-containing vesicle. Also, spontaneous EPSCs (sEPSCs, spontaneous events which arise from spontaneous vesicle release or presynaptic action potentials) can be measured in the absence of action potential blockers, and analyzed similarly to mEPSCs, although amplitude measures require the caveat that spontaneous activity can elicit multi-vesicular release and larger amplitudes. Increases or decreases in amplitude of mEPSCs or sEPSCs have proven valuable for identifying changes in postsynaptic AMPARs activity (Collingridge and Isaac 2003). There are also caveats to this method, one being that glutamate levels in a vesicle in theory could also be altered, although in practice this is considered less of an issue. Also, release mechanisms can be somewhat different for evoked release and spontaneous release (Guarnieri 2017). Another point to note is that measurement of m/sEPSCs detects release from multiple synapses, i.e., multiple inputs, to a given neuron. Thus, if a particular manipulation causes input-specific effects, such changes could be obscured in a background of multiple other unchanged inputs or oppositely affected inputs. An approach that solves this problem is the measurement of optogenetically evoked asynchronous EPSCs (asEPSCs) (e.g., Britt et al. 2012; Joffe and Grueter 2016). The replacement of calcium with strontium in the extracellular solution promotes asynchronous, quantal release of glutamate following afferent stimulation

for several hundred milliseconds (Goda and Stevens 1994). Thus, asEPSCs measured during this "after-discharge" time window can be attributed to glutamate release from the activated terminal, with differences in frequency or amplitude indicative of pre- or postsynaptic changes, respectively, for the activated input (Abdul-Ghani et al. 1996).

In addition to measuring sensitivity of AMPAR currents to a CPAR inhibitor such as NASPM, CPARs can be detected electrophysiologically by measuring the rectification index. Specifically, AMPAR currents are typically measured at a series of voltages ranging from −70 mV to +40 mV. Rectification can then be assessed, e.g., as the ratio of AMPAR currents at +40 mV versus −60 mV. If CPARs are present, then currents at positive potentials will be smaller than predicted by the linear current–voltage relationship of non-CPAR AMPARs. Thus, it is fortunate that there are clear complementary electrophysiological and pharmacological methods to identify CPARs, given that many addiction-related AMPAR changes involve the appearance of CPARs.

In addition to postsynaptic measures, greater AMPAR function could also reflect increased release of glutamate-containing vesicles from the presynaptic compartment. If larger AMPAR currents are due only to greater glutamate release, then there should be no change in indices of postsynaptic AMPAR function, such as the mEPSC amplitude or AMPAR-to-NMDAR ratio. Presynaptic vesicle release can be assessed by two measures. First, an increase in the frequency of mEPSCs is taken to indicate more spontaneous vesicle release events. Second, one can assess the probability of release using the so-called paired-pulse ratio (PPR), where two EPSCs (or IPSCs) are generated 50–100 ms apart, and the second evoked current is divided by the first. Residual calcium in the presynaptic terminals can linger after the first stimulation, leading to greater vesicle release for the second stimulation, which is known as paired-pulse facilitation. On the other hand, strong vesicle release in the first stimulation can deplete the vesicle pool, leading to a smaller current with the second stimulation, known as paired-pulse depression. Thus, decreased PPR is taken to indicate greater release probability. There are some important caveats when using these methods. While mEPSCs are easy to measure, the release mechanisms during spontaneous release and electrically evoked release can be different, with electrical release more physiologically relevant. Also, greater mEPSC frequency can actually reflect an increased number of synapses, rather than a presynaptic effect. One well-studied example of this is called "silent synapses," which contain NMDARs but not AMPARs (Collingridge and Isaac 2003; Chen et al. 2010; Neumann et al. 2016). In particular, insertion of AMPARs into these silent synapses increases AMPAR function by increasing the number of AMPAR-containing synapses, which can elevate AMPAR currents globally without increasing the number of AMPARs at any given synapse.

In addition to measures of AMPAR activity, a number of studies have examined the ability of patterned glutamatergic release to produce long-term increases or decreases in AMPAR activity, the so-called LTP or long-term depression (LTD), which could contribute to long-term storage of memories including those related to addiction. If greater AMPAR function after intoxicant exposure reflects formation of

LTP, in that it utilizes the same mechanism, then LTP induction ex vivo should be impaired (since it has already been induced). One might also expect to generate a larger LTD, since there is a larger range for AMPARs to fall from the LTP state (e.g., Ungless et al. 2001). However, a number of forms of AMPAR plasticity have been identified (Malinow and Malenka 2002; Henley and Wilkinson 2013), which may complicate interpretation of the mechanisms of a given AMPAR adaptation. Also, biochemical measures of AMPAR activation would be particularly useful, especially how they are changed by LTP/LTD inducing protocols.

Another interesting possibility is that intoxicant exposure could disrupt the signaling cascade necessary for plasticity induction, which may prevent neurons from generating new LTP or LTD (also called anaplasticity). For example, obesity in animals is associated with increased NAc AMPARs and decreased LTD induction (Brown et al. 2017). Furthermore, the ATPase Thorase facilitates internalization of AMPARs, while disrupting Thorase increases AMPAR function and impairs induction of both LTP and LTD (Pignatelli et al. 2017). In practice, it can be difficult to assess whether a given intoxicant-related "disruption" in generating LTP or LTD reflects an impairment in a signaling cascade necessary for plasticity induction, rather than an adaptation in AMPARs themselves, since both could occur at the same time. Interestingly, it is also possible that the observation that addicted humans can decrease involvement with non-intoxicant rewards (e.g., see Perry et al. 2015) reflects where repeated drug exposure impairs formation of memories for other, nondrug rewards.

In recent years, newer techniques have been developed which have significantly advanced our ability to examine changes in glutamate receptor function. Glutamate uncaging can reveal AMPAR function and rectification at the level of single spines (e.g., Lalanne et al. 2016), and can also be used to induce synaptic plasticity (e.g., Chiu et al. 2017). While these methods have not, to our knowledge, been used in relation to alcohol drinking studies, they could strongly enhance our mechanistic understanding to AMPAR adaptations that contribute to alcohol and other addiction-related behaviors.

Thus, there a number of measures of AMPAR function that serve as complementary windows into different aspects of glutamatergic signaling. In the best case, different electrophysiological measures would provide a consistent pattern. For example, a postsynaptic AMPAR increase would be evident as a larger AMPAR-to-NMDAR ratio, a larger input–output relationship for AMPAR but not NMDARs, and a larger mEPSC amplitude, with no change in mEPSC frequency or paired-pulse ratio. In contrast, increased presynaptic release would be reflected in a decrease in the paired-pulse ratio and an increase in mEPSC frequency, with no change in mEPSC amplitude or AMPAR-to-NMDAR ratio. It is important to understand the strengths, weaknesses, and interpretation of the different glutamate receptor experiments, both when performing them and when interpreting published results.

3 Regulation of AMPARs by Phosphorylation and Accessory Proteins

In addition to electrophysiology measures of AMPAR function, a number of biochemical measures for AMPARs can be utilized to indicate or infer the AMPAR activity state. A considerable amount has been discovered about regulation of AMPARs by posttranslational modifications, phosphorylation, trafficking, auxiliary subunits, and scaffolding molecules (Malinow and Malenka 2002; Collingridge and Isaac 2003; Fukata et al. 2005; Chen et al. 2010; Citri et al. 2010; Traynelis et al. 2010; Mao et al. 2011; Bats et al. 2013; Henley and Wilkinson 2013; Hanley 2014; Wang et al. 2014). Thus, in addition to electrophysiology, biochemical methods are invaluable in assessing possible AMPAR adaptations. Western blot can be used to determine the protein levels of specific AMPAR subunits in the total homogenate or in fractions concentrated for synaptic components (e.g., see Conrad et al. 2008; Beckley et al. 2016). Also, one can determine the level of AMPARs at the surface of the neuron using cross-linking methods which isolate surface proteins (Conrad et al. 2008; Mickiewicz and Napier 2011; Russell et al. 2016). In addition, recent advances in immunocytochemistry and laser-scanning microscopy allow the visualization of GluA subunits within dendrites and spines (e.g., Sebastian et al. 2013). Furthermore, antibodies are available for several regulatory phosphorylation sites (e.g., Hayashi and Huganir 2004; Chung et al. 2000). The model in Fig. 1 presents several widely studied regulatory interactions for GluA1 and GluA2 AMPAR subunits, including critical phosphorylation sites and interacting molecules that alter both AMPAR localization and function. Some regulators, including the auxiliary molecules transmembrane AMPA receptor regulatory proteins or TARPS (e.g., stargazin), promote trafficking and surface anchoring of all AMPAR subunits (Fukata et al. 2005; Jackson and Nicoll 2011; Henley and Wilkinson 2013), while other regulatory steps are specific to GluA1 or GluA2. Here we present only a subset of known molecular regulators of AMPARs, focusing on those studied thus far in the context of addiction-related behaviors. In particular, some of the described AMPAR changes are not as of yet reported for alcohol drinking, but their generation in other addiction-related behaviors means they are possible.

One could increase CPAR function by increasing GluA1 activity. Regulation of GluA1 at serine 831 (S831) and serine 845 (S845) is associated with increased levels of GluA1 and functional CPAR currents (Derkach et al. 1999; Banke et al. 2000; He et al. 2009; Park et al. 2014). In addition, scaffolding proteins such as SAP97 can stabilize GluA1 surface expression (Leonard et al. 1998; Waites et al. 2009). As we will see, all of these mechanisms that increase GluA1 activity can be associated with greater expression of addiction- and motivation-related behaviors (Park et al. 2014; Zheng et al. 2015; White et al. 2016; Cannady et al. 2017; Martinez-Rivera et al. 2017; Ouyang et al. 2017).

One could also increase CPAR function by reducing the impact of GluA2 subunits. As shown in Fig. 1, there are several amino acid residues in the GluA2 C-terminus that, when phosphorylated, promote removal of GluA2 from the synapse. In particular, GluA2 serine 880 (S880) and a tyrosine cluster just upstream of

S880 have been studied by several groups in the context of addiction-related behaviors. For example, the scaffolding protein GRIP stabilizes GluA2 at the synapse, but when S880 is phosphorylated by PKC, GluA2 is internalized due to disrupted association of GluA2 with GRIP (Chung et al. 2000; Henley and Wilkinson 2013). As described below, knocking down GRIP in the NAc increases CPAR activity and cocaine reinstatement (Briand et al. 2014). Others have developed interference peptides based on the GluA2 C-terminal sequence that target the trafficking of GluA2-containing AMPARs. One example is the $GluA2_{3Y}$ peptide that is based on the tyrosine cluster upstream of S880 (^{869}YKEGYNVYG^{877}). This peptide specifically interferes with clathrin-dependent, regulated GluA2 endocytosis, and prevents LTD without affecting basal AMPAR transmission (Ahmadian et al. 2004; Brebner et al. 2005; Choi et al. 2014). Interestingly, this peptide attenuates cue-induced reinstatement of heroin seeking and blocks cue-induced increases in CPAR activity in the mPFC (Van den Oever et al. 2008). This finding is especially noteworthy because it explicitly demonstrates that regulated GluA2 endocytosis is a critical step in drug cue-induced upregulation of CPAR activity. Infusion of this peptide into the NAc also prevents the expression of amphetamine sensitization, suggesting that NAc LTD (i.e., regulated GluA2 endocytosis) in vivo is required for this behavior (Brebner et al. 2005). Furthermore, as discussed below, studies with this peptide find disruption of alcohol consumption under some but not all circumstances. Thus, GluA2 endocytosis concomitant with upregulation of CPAR activity is a category of AMPAR adaptation that appears to be common to several classes of intoxicants.

Finally, increased AMPAR function could also reflect increased GluA2-related mechanisms. For example, alcohol drinking increases synaptic levels of GluA1 and GluA2 and AMPAR currents in the dorsal-medial striatum (Wang et al. 2012, 2015), and it would be interesting to determine whether this occurs without a switch to CPAR expression. In addition, several studies have used mice with a GluA2-K882A knockin, which, by preventing PKC action at GluA2-S880, stabilizes functional GluA2 at the synapse. These knockin mice show greater AMPAR currents in NAc neurons, but no change in the CPAR contribution, along with larger behavioral responses to stressors and stress-, cue, or cocaine-induced reinstatement of cocaine self-administration (Briand et al. 2016; Ellis et al. 2017). Thus, elevating GluA2 function, without any shift to CPARs, can also be sufficient to drive excessive behavior.

4 The Role of CPARs in Alcohol-Related Behaviors

Although there are multiple possible forms of AMPAR adaptation, we focus here initially on possible alcohol-related adaptations in CPARs, in part because they have received significant attention, and also because there is evidence that a number of identified AMPAR changes involve CPARs. Alcohol exposure leads to expression in CPAR activity in a number of brain regions, including the NAc, dorsal-medial striatum (DMS), central amygdala (CeA), ventral tegmental area (VTA), cortex, and

bed nucleus of the stria terminalis (BNST). These regions have been shown to contribute to motivation- and addiction-related behavior, with some differential roles (Chaudhri et al. 2010; Fineberg et al. 2010; Koob and Volkow 2010, 2016; Barker et al. 2015; Koob and Mason 2016; Cooper et al. 2017; Gremel and Lovinger 2017; Vranjkovic et al. 2017). In this section, we will address alcohol-related CPAR adaptations and their potential behavioral consequences; then subsequent sections will examine examples from outside of alcohol to illustrate other key points about AMPAR regulation and adaptation. Also, although many AMPAR adaptations reflect increased CPAR function, AMPAR function can be increased without the involvement of CPARs. See Fig. 2 for an overview of mechanisms implicated in ethanol-induced AMPAR adaptations.

4.1 NAc

The NAc is important for driving motivated behavior, with the core perhaps more important for action driven by discrete events (e.g., a cue), with the shell mediating conditions ranging from uncertainty and primary reward, to context and cued reinstatement (Chaudhri et al. 2010). Intermittent alcohol drinking strongly increases GluA1 protein levels in the NAc through mTORC1-dependent signaling, and inhibiting mTORC1 action in the NAc significantly decreases alcohol intake levels (Neasta et al. 2010). Since that time, transgenic mice expressing markers specifically within the dopamine D1R- or D2R-expressing medium spiny neurons (D1-cells or D2-cells) have allowed evaluation of AMPAR activity specifically within each cell type. Interestingly, a single alcohol drinking session increases mTORC1 activation and CPAR function only in D1-cells of NAc shell (Beckley et al. 2016), and not in the NAc core. This was associated with a greater AMPAR-to-NMDAR ratio and larger mEPSC amplitude, both signs of increased AMPAR activity, and also with greater rectification, which reflects the presence of functional CPARs. Thus, GluA1- and CPAR-related increases in the NAc shell likely drive excessive alcohol drinking.

In strong concurrence, repeated, intermittent exposure to alcohol vapor also leads to CPAR expression in D1-cells of the NAc shell (Renteria et al. 2017). In addition, D1-cells actually show a constellation of alcohol-related adaptations, while NAc D2-cells show fewer such adaptations. Following alcohol vapor-exposure, D1-cell AMPARs show greater inward rectification than observed in air-treated controls, indicating the presence of CPARs. However, the amplitude of sEPSCs was not changed by alcohol vapor. Thus, these data suggest a change in the balance of GluA1 and GluA2 subunits, without a change in total postsynaptic AMPARs, after alcohol vapor. In addition, measures of presynaptic release, including both spontaneous EPSC frequency and paired-pulse ratio, were greater after alcohol vapor, suggesting there may be both pre- and postsynaptic adaptations in glutamatergic function in the NAc shell after repeated alcohol vapor. In contrast, we did not see changes in AMPAR to NMDAR ratio, or several other measures of AMPAR function, in glutamatergic inputs to the NAc core after long-term intermittent alcohol intake in

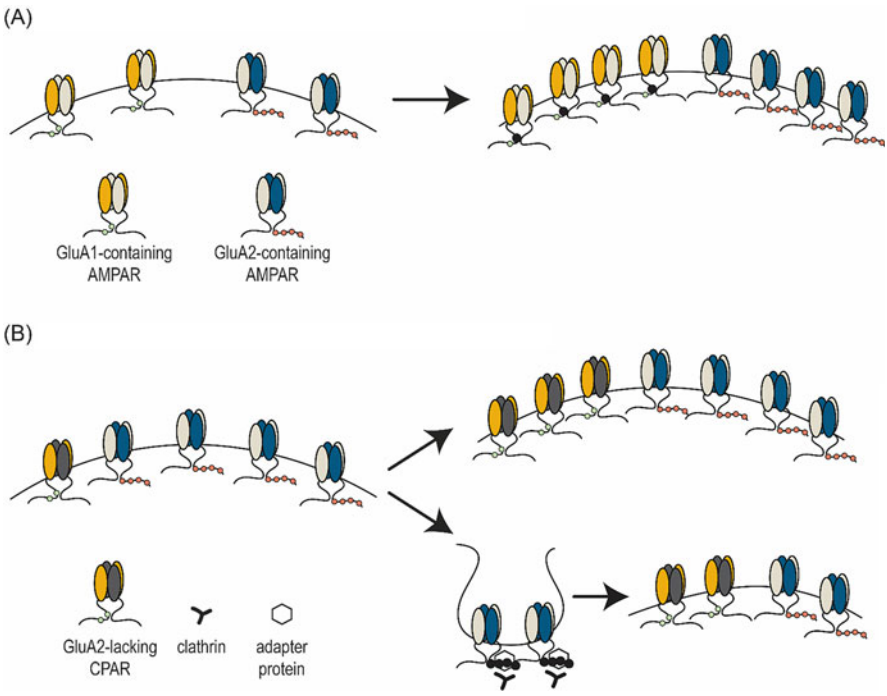

Fig. 2 Simplified illustration of possible ethanol-induced AMPAR adaptations. The left side of each panel represents an ethanol-naïve state, with the right side depicting possible ethanol-induced AMPAR changes. (**a**) Increases in AMPAR subunit expression or function that are associated with enhanced overall AMPAR function. GluA1 subunits are yellow; GluA2 are blue; ivory subunits represent where the identity of the other two subunits may vary. Phosphorylated residues (described in Fig. 1) are enlarged and filled in black. Elevated GluA1 S831 phosphorylation and/or increased total, surface, or synaptic GluA1 protein expression is observed in the OFC, DMS, and subregions of the amygdala and NAc. In some studies, effects on other AMPAR subunits were not evaluated (Neasta et al. 2010; Salling et al. 2016; Cannady et al. 2017; Nimitvilai et al. 2017). In others, these changes in GluA1 are observed alongside increased surface or total GluA2 protein (Christian et al. 2012; Wang et al. 2012; Nimitvilai et al. 2016). (**b**) Increases in the contribution of CPARs, with or without an increase in overall AMPAR transmission. Elevated contribution of CPARs is documented in the VTA and subregions of the NAc. The top pathway illustrates situations in which the increased CPAR contribution enhances AMPAR transmission overall (Marty and Spigelman 2012; Hausknecht et al. 2015; Beckley et al. 2016), while the bottom pathway illustrates conditions of GluA2 internalization where CPARs increase without a change in total AMPAR transmission (Renteria et al. 2016a, 2017). Color scheme is the same as in (**a**), with the addition of dark gray in GluA1-containing AMPARs to indicate where the subunit cannot be GluA2

rats (Hopf et al. 2010a, unpublished findings), although we did not specifically look for CPAR function.

In addition to changes in AMPAR composition after chronic alcohol experience, NAc neurons also show profound changes in the ability to express glutamatergic synaptic plasticity (Abrahao et al. 2013; Jeanes et al. 2011; 2014; Ji et al. 2017;

Spiga et al. 2014; Renteria et al. 2016a, b, 2017). For example, NMDAR-dependent LTD of AMPAR-mediated EPSCs can be induced in D1-cells, but not D2-cells, in the NAc shell and core in alcohol naïve mice. However, after alcohol vapor exposure, NAc shell D1-cells exhibit no LTD, or even LTP, while D2-cells exhibit LTD (Jeanes et al. 2014; Renteria et al. 2016b, 2017). These changes are accompanied by an increase in NMDAR currents in D1-cells and a decrease in NMDAR currents in D2-cells (Renteria et al. 2016b). Renteria et al. (2017) found that the metaplastic effects of alcohol exposure on D1-cells were specific to the NAc shell, and a similar distinction between NAc shell and core D1-cells also was observed by Mangieri et al. (2017). After operant ethanol self-administration experience, the magnitude of LTD in NAc shell, but not core, D1-cells was inversely correlated to prior ethanol consumption, with the highest-drinking mice showing normal LTD in core D1-cells, but no LTD at all in shell D1-cells. On the other hand, Abrahao et al. (2013) found that NMDAR-dependent AMPAR-mediated LTD in unidentified NAc core neurons was impaired during withdrawal from chronic ethanol treatment, but only in mice that had shown sensitization to the locomotor-stimulating effects of ethanol. Notably, sensitized mice also voluntarily consume more ethanol compared to non-ethanol-treated or ethanol-treated, non-sensitized mice. Thus, although the specific ethanol-induced changes in NAc glutamatergic synaptic plasticity appear to vary by several factors, including subregion, cell type, and model of exposure, the findings of Abrahao et al. and Mangieri et al. suggest there may be a general relationship between individual differences in ethanol-induced metaplasticity and individual differences in ethanol consumption.

An important question is the relationship between the disappearance or impairment of LTD and the appearance of CPARs in NAc cells following alcohol exposure, and, moreover, the relevance of these changes to alcohol-related behaviors. One possibility is that the presence of CPARs reduces the stimulus intensity threshold required for inducing LTP rather than LTD, since the same level of stimulation would produce greater rises in intracellular Ca^{2+} during the plasticity induction stimulus. Only one of the studies cited in the preceding paragraph (Renteria et al. 2017) specifically tested for changes in CPAR function, however. Thus, it remains to be exhaustively demonstrated that a loss or reduction in the ability to induce LTD after drug or alcohol exposure is a reliable indicator of the presence of CPARs, and vice versa.

Some insight as to the relevance of CPARs and LTD to alcohol and other reward-related behaviors comes from experiments in which endocytosis of GluA2-containing AMPARs is disrupted. As discussed in the preceding section, the $GluA2_{3Y}$ peptide prevents LTD induction by interfering with the regulated endocytosis of GluA2-containing AMPARs (CIARs) and, at least under some conditions (e.g., Van den Oever et al. 2008), thereby blocks upregulation of CPAR activity. Thus, presumably, this peptide could prevent replacement of CIARs by CPARs in NAc D1-cells – although to our knowledge this remains to be tested explicitly. In a study by Lim et al. (2012), chronic restraint stress promoted CPAR activity in NAc core D1-cells and decreased sensitivity to food and cocaine reward. Blocking endocytosis of GluA2-containing AMPARs (with a peptide that works similarly to

$GluA2_{3Y}$) in the NAc prevented the chronic stress-induced decreases in reward sensitivity (Lim et al. 2012), without affecting reward sensitivity in non-stressed mice. Similarly, expressing the $GluA2_{3Y}$ peptide in the NAc does not appear to affect alcohol intake under conditions in which low to moderate doses of ethanol are consumed (e.g., operant self-administration), but it does prevent the chronic, intermittent, alcohol vapor-induced escalation of non-operant ethanol consumption (Renteria et al. 2016a; Maier et al. unpublished observations). Unfortunately, none of this work in the NAc directly assessed whether disruption of GluA2 endocytosis prevents upregulation of CPARs. However, taken together, these findings are consistent with the idea that GluA2-containing CIARs are endocytosed and replaced by CPARs in the NAc as a result of strong physiological challenges and that this phenomenon specifically underlies behavioral modification in the face of such challenges.

4.2 DMS

The DMS has been considered crucial for goal-directed behaviors, perhaps the counter to DLS-driven habitual behavioral drives. Enhanced cannabinoid signaling after alcohol exposure inhibits mPFC-DMS inputs, facilitating habitual responding (Gremel and Lovinger 2017). Also, AMPAR adaptations in the DMS can drive excessive alcohol drinking. Both alcohol exposure in the slice and alcohol drinking in vivo, which are known to increase DMS NMDAR function, also enhance AMPAR signaling in a form of LTP (Wang et al. 2012). This is accompanied by increased protein levels of GluA1 and GluA2 in the synaptic membrane fraction. Additional studies (Wang et al. 2015) indicate that this alcohol-related enhancement in AMPAR function occurs in D1-cells but not D2-cells in the DMS, which is accompanied by cell-type-specific structural plasticity. Furthermore, recent work (Ma et al. 2017) shows that excessive alcohol drinking leads to increased AMPAR function at mPFC-DMS inputs, while the probability of glutamate release is elevated at amygdala inputs. Furthermore, co-activation of mPFC and amygdala inputs leads to LTP only of the mPFC inputs to DMS. Finally, inhibiting AMPARs (Wang et al. 2012) or D1 (but not D2) receptors (Wang et al. 2015) within the DMS decreased operant alcohol but not sucrose self-administration. Thus, alcohol-related, projection-specific AMPAR changes in the DMS promote excessive alcohol intake. To our knowledge, the contribution of CPARs to DMS AMPAR plasticity has not yet been investigated, but remains an area of great interest, especially if the DMS is a case of non-CPAR alcohol-related AMPAR plasticity.

4.3 DLS

The DLS is important for habit-driven behavior, and is likely a critical contributor to excessive alcohol intake and more pathological forms of drinking such as compulsion. Intermittent alcohol vapor impairs generation of synaptic plasticity in the DLS (DePoy et al. 2015), and DLS AMPARs are required for expression of

alcohol-related habit (Corbit et al. 2014). To our knowledge, DLS AMPAR function in relation to alcohol has not been directly investigated.

4.4 CeA

An interesting line of research has shown that moderate, voluntary intake through operant self-administration leads to AMPAR adaptations in the CeA (Salling et al. 2014, 2016; Cannady et al. 2017). Alcohol intake increases phosphorylation of GluA1 at S831 in the amygdala, in agreement with alcohol vapor increasing amygdala GluA1 surface levels (Christian et al. 2012). In addition, calmodulin kinase II (CMKII) is known to phosphorylate GluA1-S831, and intra-CeA inhibition of AMPARs or CMKII decreases alcohol but not sucrose self-administration (Salling et al. 2016; Cannady et al. 2017). Further, a positive allosteric modulator of AMPARs increases alcohol intake when infused in the CeA, and this effect is blocked by a subthreshold level of CaMKII inhibitor. While these studies did not look for CPARs directly, increased phosphorylation at GluA1-S831 can be associated with greater CPAR activity (Park et al. 2014). Together, these results suggest that moderate voluntary alcohol intake can increase GluA1 and CPAR levels in the CeA, which contributes to maintaining ongoing self-administration. Interestingly, CeA AMPARs are also implicated in learning about opiate reward (Cai et al. 2013), which concurs with the idea that the CeA regulates the moderate alcohol drinking that may be more related to the primary rewarding effects of alcohol.

These findings are also interesting in the context of findings that the CeA is a central contributor to the "dark side" of alcohol addiction, where intake is thought to occur in order to relieve negative consequences (Koob and Mason 2016). The CeA has been shown to drive the greater intake after induction of dependence in rats, in part through CRF-related adaptations (e.g., Funk et al. 2006). This same CRF-related adaptation drives the greater binging after 4 days of drinking-in-the-dark in mice (Lowery-Gionta et al. 2012). Thus, it is interesting that increased AMPARs in CeA could also be protective against excessive drinking, but also that, in a different experimental context, CeA activity is important for driving excessive intake. This could also reflect where the CeA has complex internal anatomy, with GABA cells connected in series that can disinhibit behaviors (Johansen et al. 2012). Thus, CeA AMPAR contributions may reflect signaling within specific CeA cell types, and thus could have very different effects on behavior.

4.5 VTA

Psychostimulants increase VTA CPAR function (Luscher 2013, 2016), and thus there is rationale for alcohol intake having a similar effect. Intermittent alcohol drinking increases AMPAR synaptic strength in the VTA (Stuber et al. 2008), but the possible role of CPARs remains untested. Interestingly, fetal alcohol exposure is associated with increased CPARs in the VTA (Hausknecht et al. 2015). Not only did

this increase self-administration of amphetamine, it also led to the appearance of a CPAR-dependent LTP, where enhanced CPAR signaling led to further increases in VTA AMPAR activity.

4.6 Cortex

Given the importance of cortical areas for behavioral control, AMPAR adaptations in these regions could profoundly alter the expression of behavior (e.g., Otis and Mueller 2017). While repeated alcohol vapor does not change mPFC AMPAR protein levels in rat (Trantham-Davidson et al. 2017) or mouse (Kroener et al. 2012), increasing the level of CPARs in the infralimbic but not prelimbic mPFC was protective against alcohol reinstatement (Gass et al. 2014). CPAR levels in mPFC were elevated using a positive allosteric modulator of mGluR5, although total AMPAR function was not increased. Thus, a switch from CIARs to CPARs in the ventral mPFC could actually be protective against alcohol relapse. However, mPFC CPARs mediate behavioral sensitization after nerve injury (Chen et al. 2014), indicating that cortical areas still have the potential to express long-term CPAR adaptations which potently regulate negative aspects of behavior.

While repeated alcohol does not seem to alter mPFC AMPAR currents, alcohol drinking leads to significant AMPAR changes in the orbitofrontal cortex (OFC) in both monkeys and mice (Nimitvilai et al. 2017). Heavy-drinking monkeys show greater GluA1 expression and larger amplitude of spontaneous EPSCs in OFC, both suggestive of increased AMPAR function. In addition, repeated alcohol vapor increased the AMPAR-to-NMDAR ratio in lateral OFC, with an increase in the ratio of GluA1 to GluA2 expression, as well as facilitated LTP induction (Nimitvilai et al. 2016). The increase in GluA1 levels relative to GluA2 in OFC may suggest that the increased OFC AMPAR function reflects a shift towards CPARs, although this remains to be directly tested. Nonetheless, it is clear that alcohol enhancement of cortical AMPARs likely strongly promotes heavy alcohol drinking.

4.7 BNST

Alcohol vapor exposure alters some forms of AMPAR LTD in the BNST (McElligott et al. 2010). BNST neurons exhibit two forms of LTD, one mediated through mGluR5s and the other through α1-adrenergic receptors. Interestingly, the adrenergic-mediated LTD involves signaling through CPARs, and is disrupted by chronic alcohol or stress exposure, while mGluR-mediated LTD is not. While there is more to understand about how alcohol regulates BNST AMPARs, these findings suggest that alcohol exposure could cause more selective effects on stress responding mediated through the BNST.

5 Instructive Examples of Other CPAR and Non-CPAR Adaptations

Much has been learned about the ability of intoxicants other than alcohol to induce CPAR and other AMPAR adaptations within brain regions including the NAc and VTA (Pickens et al. 2011; Pierce and Wolf 2013; Luscher 2013, 2016). In addition, different types of passive and active exposure can produce differential effects (e.g., Martin et al. 2006; Chen et al. 2008; Terrier et al. 2016). On the one hand, these studies represent particular AMPAR changes that could develop. However, cocaine/psychostimulants can act through different circuitry from other intoxicants (Marchant et al. 2015), and might be predicted to have different AMPAR and signaling mechanisms (e.g., Karoly et al. 2015; Graziane et al. 2016; see below). Common and divergent mechanisms among intoxicants are equally interesting and informative.

Considerable work has examined CPAR expression in the NAc that develops slowly across protracted abstinence from repeated cocaine exposure, which has been called incubation of cocaine craving (Conrad et al. 2008; Wolf and Ferrario 2010; Pierce and Wolf 2013). There are also more complex changes in AMPAR function early in abstinence (Ortinski et al. 2012). Cocaine incubation has been associated with greater surface expression of GluA1, decreased surface levels of GluA2, and electrophysiological indicators of CPARs, and with little evidence for NAc CPARs in control animals. In addition, cocaine seeking is significantly reduced after infusing the CPAR inhibitor NASPM into the NAc. Importantly, food-seeking control animals do not show increased NAc CPARs, and their food seeking is not altered by NASPM in the NAc. Also, GluA2 pre-mRNA can be edited by a protein called ADAR, where unedited GluA2 actually contribute to CPARs (see Schmidt et al. 2015). Under normal conditions, the vast majority of GluA2 is edited, resulting in GluA2-containing CIARs. In contrast, withdrawal from cocaine intake reduces ADAR and increases the number of unedited GluA2 in the NAc shell, which is associated with the presence of CPARs (Schmidt et al. 2015). In addition, increasing ADAR levels reduced reinstatement. Thus, there is considerable evidence that increased NAc CPARs drive high relapse after protracted abstinence, which in part reflects an increase in CP-GluA2 subunits.

Other interesting findings have been observed after cocaine self-administration and extinction, and in relation to reinstatement of seeking. Sutton et al. (2003) identified an increase in NAc AMPARs during extinction, and provided evidence that this increase was protective against reinstatement. In addition, cocaine self-administration and extinction have been recently shown to increase mTORC1 activity and synaptic GluA1 and CaMKII levels in the NAc shell and core (James et al. 2014). While mTORC1 inhibition does not decrease cocaine self-administration, intra-NAc shell mTORC1 inhibition decreases cued reinstatement, progressive ratio, extinction responding, and the enhanced NAc GluA1 and CaMKII levels observed after cocaine.

Other very interesting studies have shown that cue induction of reinstatement in extinguished cocaine-seeking animals can rapidly enhance AMPAR function in the NAc core (Shen et al. 2014; Gipson et al. 2013a). The NAc core AMPAR-NMDAR

ratio was increased after extinction from cocaine, and reinstatement enhanced AMPAR function further. This increased AMPAR function and the associated greater spine growth can be instated and retracted quickly (Kalivas and Kalivas 2016). Similar rapid increases in NAc AMPAR function and GluA1 levels are also observed after nicotine self-administration (Gipson et al. 2013b). Thus, not only can cocaine exposure enhance NAc AMPAR function, exposure to cocaine cues can rapidly induce AMPARs, which likely contribute to the expression of reinstatement. We also note that, in contrast to cocaine, heroin cues decrease mPFC GluA2 levels and AMPA-NMDAR ratios (Van den Oever et al. 2008; see also Cruz et al. 2008; Glass et al. 2008). This supports the idea that psychostimulants act through different mechanisms from opiates, which could in part reflect that strong acute drugs (such as cocaine and perhaps nicotine) act differently from more diffuse rewards (such as opiates and alcohol).

Also noteworthy is a seeming consensus that a number of forms of challenge, ranging from alcohol intake to stress and novelty, increase NAc CPARs predominantly within D1-cells, with little basal AMPAR change in D2-cells, and primarily within the NAc shell rather than NAc core. These studies are mainly carried out in mice, where transgenic animals expressing fluorescent proteins in D1- or D2-cells allow dissection of cell-type-specific AMPAR activation. As mentioned above, CPAR induction only in D1-cells is observed in the NAc shell after alcohol drinking (Beckley et al. 2016) and after alcohol vapor (Renteria et al. 2017). Increases in CPARs within D1- but not D2-cell of the NAc shell have also been reported after exposure to cocaine (Terrier et al. 2016), opiates (Hearing et al. 2016; Russell et al. 2016), and food deprivation (Ouyang et al. 2017; see also Oginsky et al. 2016 for junk food diet). Interestingly, both alcohol and cocaine enhancement of NAc shell CPARs are dependent on mTORC1 signaling (James et al. 2014; Beckley et al. 2016). Furthermore, inhibiting mTORC1 signaling in the NAc suppresses alcohol drinking (Neasta et al. 2010) and cocaine seeking (James et al. 2014), reinforcing the behavioral importance of elevated NAc CPARs. Finally, morphine CPP is also associated with increased CPARs only in NAc shell D1-cells (Hearing et al. 2016), and treatments that reverse CPAR expression also prevent morphine reinstatement. Changes in AMPARs across a variety of intoxicants have also been observed in the VTA (Saal et al. 2003; Luscher 2013). This convergence across intoxicants and exposure methods is one reason that we focus on CPAR adaptations and the critical importance of NAc mTORC1 signaling cascades in AMPAR regulation of problem alcohol drinking and cocaine intake.

While rat and mouse studies both implicate NAc CPAR adaptations during addiction-related behaviors, there seems to be a fundamental conundrum. In studies from rats, increased CPAR function is typically observed across most of the NAc cells examined. In contrast, results from mice would predict that only half of rat NAc cells should show the AMPAR adaptation, corresponding to a change in D1-cells but not D2-cells. There are certainly technical differences across rat and mouse studies, including the level of intake. However, dorsal striatal D2-cells in mice have about twice the intrinsic excitability (the number of action potentials fired for a given level of depolarizing current) relative to mouse D1-cells. In contrast, the intrinsic excitability of dorsal striatal neurons in rat is very similar across all cells (Hopf et al. 2010b), with no

evidence for two populations of cells as would be predicted from mouse studies. Also, some mouse–rat projection-specific differences are observed in the VTA (Margolis et al. 2008; Lammel et al. 2012). While we must collectively reckon with such species discrepancies, these examples also underscore the critical importance of examining the impact of a molecular adaptation on behavior in both rat and mouse. This will greatly increase one's confidence in the translational relevance of one's pathway.

While CPARs, both in NAc shell and in general, may reflect a fundamental, common mechanism that can regulate excitability and behavior, it is important to note that adaptations in AMPARs other than CPARs also likely play a critical role under some behavioral conditions. Increased NAc glutamatergic transmission and cocaine reinstatement are observed after preventing GluA2 phosphorylation at S880 which normally leads to GluA2 internalization into the cell, or by overexpressing GluA2 (Briand et al. 2016; Ellis et al. 2017). In addition, stabilization of surface GluA2 levels and increased GluA2 function in the K882A knockin mouse have been associated with increased susceptibility to social defeat stress (Ellis et al. 2017), while GluA2 overexpression in NAc increases resilience to social defeat (Vialou et al. 2010). Thus, increased CPARs may increase reactivity for intoxicants and cues at the cost of decreasing resilience to stress. In addition, K882A knockin mice do not show a change in inward rectification, while GluA2 overexpression by viral methods is associated with decreases in rectification, suggesting a reduction in CPARs. While some differences may relate to technical matters, including global versus local modulation of GluA2 function, it also underscores the possibility that AMPARs are regulated by a dynamic system whose regulation may be complex.

We would expect that some behaviors will not require NAc CPARs, either because CPARs are not induced by that behavior, or because CPARs are present but the NAc is not important for expression of the given behavior. For example, behavioral flexibility can require NAc core and shell NMDAR signaling, but with no role for CPARs in either region (Ding et al. 2014). One interesting possibility is that behaviors only become dependent on CPAR when there are adaptations associated with increased CPAR levels. This would concur with a lack of CPAR adaptations and CPAR contribution to behavior in several control behaviors including seeking of natural rewards (Conrad et al. 2008), and that CPARs are hardly present at baseline in many areas (Conrad et al. 2008; Reimers et al. 2011; Ding et al. 2014). In fact, by identifying the types of training and experience that induce CPAR expression, we may be able to better understand the central ethological role of the mechanisms that enhance CPAR function. In addition, we consider it important to examine how a given alcohol-related neuro-adaptation might alter intake of a natural reward (Seif et al. 2013, 2015), in contrast to having controls where separate animals receive the natural reward but never consume alcohol. These animals never develop the CPAR adaptation, so it is unlikely that CPARs would promote reward-related behavior. In contrast, the CPAR adaptation in alcohol drinkers still has the possibility to influence natural reward intake, and should be tested directly.

Finally, while classic electrophysiological techniques have identified more global changes in AMPAR function, a number of exciting recent studies have used optogenetics ex vivo and discovered that specific inputs into a given brain region can actually have different types of AMPAR adaptations. In particular, a specific

projection is often labeled with the excitatory channelrhodopsin, so that light can be used to release glutamate only from that projection, allowing determination of input-specific glutamate receptor signaling (see also Lane et al. 2008; Good and Lupica 2010). For alcohol, this has been shown for the NAc core (Seif et al. 2013) and the DMS (see above). Cocaine exposure also leads to complex projection-specific glutamate receptor adaptations within the NAc, measured across inputs from mPFC, amygdala, paraventricular thalamus, and hippocampus (Britt et al. 2012; Pascoli et al. 2014; Joffe and Grueter 2016; Neumann et al. 2016; Terrier et al. 2016). Projection-specific AMPAR adaptations onto NAc D2-cells also mediate opiate withdrawal (Zhu et al. 2016). Finally, the ventral hippocampus has a particularly strong glutamatergic input to the NAc shell, relative to other inputs (Britt et al. 2012), and adaptations in this input could profoundly alter expression of reward behavior.

6 Reversal of AMPAR Adaptations

A number of advances have been made in reversing enhancements in AMPAR function after intoxicant exposure. First, some forms of LTP of AMPAR function can be reversed by patterns of stimulation that induce LTD. This has been termed metaplasticity, where LTP and LTD are viewed as being opposite functional states, and where further patterned stimulation could quickly switch LTP to an LTD or vice versa. LTD-like patterned simulation in vivo can reverse several AMPAR adaptations including the elevated CPARs after cocaine or morphine (Pascoli et al. 2014; Hearing et al. 2016). Importantly, as mentioned above, the advent of channelrhodopsin has allowed activation of only specific glutamatergic inputs by LTD-inducing stimulation. It is perhaps remarkable that even a relatively brief in vivo LTD-inducing pattern can persistently reverse NAc AMPAR adaptations and their behavioral impact, as demonstrated in studies of opiates (Hearing et al. 2016; Zhu et al. 2016) and cocaine (Stefanik et al. 2016). For example, in the study from Hearing et al. (2016), optogenetic LTD of mPFC-NAc shell inputs in vivo decreases both the morphine-related increase in AMPAR to NMDAR ratio and the reinstatement of morphine CPP.

A different strategy is based on pharmacological agents that normalize a hyperglutamatergic state after intoxicant exposure. For example, cocaine reinstatement increases AMPARs, and reinstatement and the AMPAR changes are prevented by inhibiting the prelimbic mPFC (Gipson et al. 2013a). In addition, cocaine induces dysfunction in glial glutamate transport and excessive glutamate release in the NAc, which results in mGluR5 activation of NOS interneurons, glial changes, and increased NAc AMPARs and spine density, and these changes are rectified by N-acetylcysteine (Kalivas and Kalivas 2016). Thus, N-acetylcysteine normalizes the excessive glutamate release and reduces cocaine reinstatement. In addition, cocaine intake causes LTP and LTD deficits in the NAc, and N-acetylcysteine restores both through increasing mGluR2/3 activity for LTP and mGluR5 for LTD (Moussawi et al. 2009; see also Kasanetz et al. 2013). Indeed, direct or indirect

activation of mGluRIs (mGluR1/5) can decrease the effects of cocaine incubation and sensitization on cocaine seeking and AMPARs (Bellone and Luscher 2006; McCutcheon et al. 2011; Halbout et al. 2014; Jedynak et al. 2016). In contrast to mGluR normalization of AMPAR adaptations, alcohol reinstatement is suppressed by a positive allosteric modulator of mGluR5, which acts by increasing CPAR function in specific mPFC areas (Gass et al. 2014). Finally, mGluRs can play a direct role in induction of a number of forms of LTD (Henley and Wilkinson 2013; Lodge et al. 2013). Thus, there may be fundamental differences in how mGluR regulation of AMPARs impacts alcohol versus cocaine intake. Nonetheless, the general principle of mGluR agonists to treat addiction is supported by these studies, although there may be some difference among intoxicants. In addition, other agents such as ceftriaxone, which is thought to increase glial glutamate uptake, might reflect translational inroads to normalize dysfunction in glutamate signaling (e.g., Alhaddad et al. 2014; Hearing et al. 2016).

A final interesting possibility is that addiction-related, long-term increases in CPARs represent an immature form of memory. In particular, LTP can be mediated by an early increase in CPARs, where calcium influx through CPARs then causes GluA2s to traffic to the membrane to replace CPARs and form a more stable long-term memory (Liu and Cull-Candy 2000; Henley and Wilkinson 2013; Wu et al. 2017). In addition, these studies suggest that CPARs are more labile in the face of depotentiating (LTD-like) stimuli, compared with GluA2-mediated LTP which is more resistant to depotentiation. One speculation is that CPAR increases are designed to be a temporary form of memory storage, and thus could be more easily unlearned under conditions where well-ordered information is used to update associations that need to become memorized. In contrast, intoxicant exposure is likely to be long-lasting and less discrete, although it remains unclear why and how this might result in trapping of these intoxicant memories in a CPAR-dependent state.

7 Summary and Conclusions

A great deal is now understood about mechanisms that can alter AMPAR function and promote learned behaviors such as addiction. However, there is still much to learn about the specific nature of these adaptations, including the specific effect of AMPAR changes on firing activity, and the impact of AMPAR adaptations in particular cell types and projections. While much is known about the presence of alcohol-related AMPAR changes that promote intake, it is clear that there are many subtleties and possible mechanisms for AMPAR regulation. In the search for better treatments for AUD, it would be valuable to continue to expand our understanding of how specific regulatory AMPAR interactions drive pathological drinking and seeking of alcohol. Also, one important caveat for this review is that the predominance of NAc CPARs studies is not meant to imply they are the most important behaviorally. Instead, this may simply reflect where AMPAR adaptations through other mechanisms and in other brain regions have not received as much attention. Nonetheless, it is also clear

that AMPAR adaptations, including those mediated by CPARs, occur in a number of brain regions and promote excessive drive for alcohol. Thus, future studies should also seek to understand the collective effect of AMPAR changes across brain circuits on promoting problem drinking, and how they might interact with non-AMPAR adaptations (e.g., Heinsbroek et al. 2017).

Acknowledgments This study was supported by AA017072 (F.W.H.), AA016651, and AA015167 (R.A.M.). We thank Richard Morrisett for his critical review of the manuscript.

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Molecular, Neuronal, and Behavioral Effects of Ethanol and Nicotine Interactions

Paul M. Klenowski and Andrew R. Tapper

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Abstract

Ethanol and nicotine can modulate the activity of several neurotransmitter systems and signalling pathways. Interactions between ethanol and nicotine can also occur via common molecular targets including nicotinic acetylcholine receptors (nAChRs). These effects can induce molecular and synaptic adaptations that over time, are consolidated in brain circuits that reinforce drug-seeking behavior, contribute to the development of withdrawal symptoms during abstinence and increase the susceptibility to relapse. This chapter will discuss the acute and chronic effects of ethanol and

P. M. Klenowski · A. R. Tapper (✉)
Department of Neurobiology, Brudnick Neuropsychiatric Research Institute, University of Massachusetts Medical School, Worcester, MA, USA
e-mail: andrew.tapper@umassmed.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_89

nicotine within the mesolimbic reward pathway and brain circuits involved in learning, memory, and withdrawal. Individual and common molecular targets of ethanol and nicotine within these circuits are also discussed. Finally, we review studies that have identified potential molecular and neuronal processes underlying the high incidence of ethanol and nicotine co-use that may contribute to the development of ethanol and nicotine co-addiction.

Keywords
Acute drug exposure · Chronic drug exposure · Dopamine · Ethanol · Nicotine · Nicotinic acetylcholine receptors

1 Introduction

The co-use of alcohol and nicotine has remained high despite the decline of cigarette use in society (Bobo and Husten 2000). Reports estimate that between 70 and 80% of alcohol use disorder patients are smokers (Bobo 1992; Bobo and Husten 2000; Miller and Gold 1998). Additionally, smokers have an increased risk of developing alcohol use disorders (DiFranza and Guerrera 1990; Grant et al. 2004). Both drugs are amongst the leading causes of preventable death (World Health Organization 2014, 2015) and are associated with increases in cardiovascular and lung diseases (Benowitz 2003) and some forms of cancer (Sasco et al. 2004). Moreover, the co-use of alcohol and nicotine can increase susceptibility to certain forms of cancer compared to the risk posed by the drugs individually (Room 2004).

With the economic burden of addiction-related illnesses in the US rising to over $420 billion per year (Office of the Surgeon General 2016), there remains a critical need to develop improved treatment strategies for alcohol and nicotine co-dependence and to increase awareness of the harmful effects of alcohol and nicotine co-use. This requires a fundamental understanding of the overlapping neural circuitry that reinforces the rewarding properties of alcohol and nicotine co-use, and determining how repeated, long-term consumption produces neuronal adaptations that increase consumption, facilitate the transition to dependence, and increase susceptibility to relapse. In this chapter we will compare and contrast the molecular, neuronal, and behavioral effects of acute versus repeated and/or chronic alcohol and nicotine co-use and discuss the implications of these findings to the development of improved therapies aimed at reducing alcohol and nicotine co-addiction.

2 Acute Effects of Ethanol and Nicotine on Brain Reward Circuitry

As with all addictive drugs, the rewarding actions of ethanol and nicotine in the brain converge on the mesolimbic dopaminergic system. Modulation of the mesolimbic pathway by ethanol and nicotine involves several neurotransmitter systems including dopamine (DA), acetylcholine, GABA, glutamate, serotonin, and opioids (Balfour 2009; Koob 2014).

2.1 Overlapping Molecular Targets of Ethanol and Nicotine: Nicotinic Acetylcholine Receptors

Although nicotine acts more specifically than ethanol to elicit its positive reinforcing effects, both share a common receptor target, namely, nicotinic acetylcholine receptors (nAChRs). These receptors are pentameric ligand-gated cation channels that contain a combination of α and β subunits. In humans, a total of 11 subunits have been identified (α2-α7, α9, α10, β2-β4) that can form several homomeric or heteromeric conformations with varying pharmacological properties (reviewed in Gotti et al. 2007; Klink et al. 2001). In an active state, nAChRs undergo a conformational change that allows monovalent and divalent cations including K^+, Na^+, and Ca^{2+} to diffuse across the plasma membrane (Unwin 2003). This can lead to a multitude of cellular responses including membrane depolarization, modulation of intracellular signalling pathways, and neurotransmitter release (Dajas-Bailador and Wonnacott 2004).

Nicotine, the tertiary alkaloid found in tobacco, is a high-affinity agonist that binds to nAChRs and causes a conformational change in the receptor that induces an open or "active" state, allowing the flow of cations down their electrochemical gradient through the channel (Changeux et al. 1998). Ethanol, on the other hand, modulates the activity of nAChRs, not as a direct agonist, but as an allosteric modulator, likely stabilizing specific channel conformations (Forman and Zhou 1999; Zuo et al. 2004). Rapid desensitization of nAChRs occurs in the presence of nicotine and this process is thought to play an important role in modulating DA levels within the mesolimbic system. Evidence is also accumulating to suggest balance between the activation and desensitization of nAChRs caused by nicotine could be altered in the presence of ethanol, which might induce adaptations that contribute to the high incidence of their co-use (see Sect. 2.3).

2.2 Ethanol and Nicotine Modulation of Dopaminergic Activity via Nicotinic Acetylcholine Receptors

The distribution of nAChRs within the mesolimbic system is well characterized, having been shown to exist in pre-, post-, and extra-synaptic domains within the ventral tegmental area (VTA) (Feduccia et al. 2012; Hendrickson et al. 2013). The VTA is densely innervated by synaptic inputs containing nAChRs which are sensitive to ethanol and nicotine, including glutamatergic afferents from the prefrontal cortex (PFC) and cholinergic and GABAergic afferents from the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus (Champtiaux et al. 2003; Gotti et al. 2007; Mansvelder and McGehee 2000; McDaid et al. 2016) (Fig. 1). Additionally, nAChRs are highly expressed in both GABAergic and DAergic VTA neurons in addition to DAergic terminals in the NAc, and display cell-type and regionally distinct expression profiles (Hendrickson et al. 2013, Fig. 1). Both indirect and direct mechanisms of ethanol and nicotine-induced DA release have been proposed, which occurs via increased cell excitability and burst firing of VTA DAergic neurons (Chatterjee and Bartlett 2010; Hendrickson et al. 2013). Previous studies have demonstrated that nAChRs containing the β2 subunit in combination with the α4 and/or α6 subunits mediate the rewarding

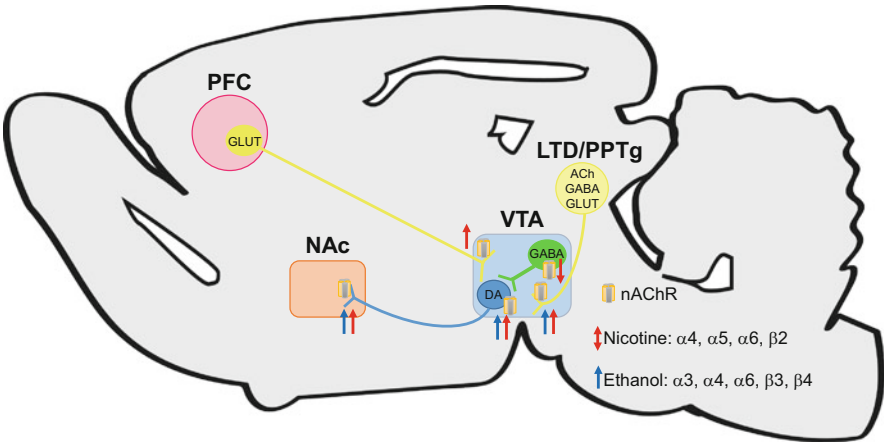

Fig. 1 Overlapping molecular targets of ethanol and nicotine in brain reward circuitry. The acute pre- and post-synaptic effects of ethanol and nicotine converge on the mesolimbic dopaminergic system. Ethanol and nicotine via nAChRs modulate glutamatergic input from brain areas including the prefrontal cortex (PFC) and laterodorsal tegmental nucleus (LTD). *Arrows* indicate ethanol and nicotine-induced increases or decreases in various reward signalling pathways including cholinergic and GABAergic afferents from the pedunculopontine tegmental nucleus, (PPTg) as well as local ventral tegmental area (VTA) GABAergic neurons and VTA DAergic neurons via pre- and post-synaptic nAChRs, leading to increased DA release in the nucleus accumbens (NAc). Also shown are nAChR subunits that are expressed within these brain regions that are involved in ethanol and nicotine reward

properties of nicotine and nicotine-induced increase in accumbal DA (De Biasi and Dani 2011). This is based on reports highlighting the absence of nicotine-induced DA release in α4 and β2 knock-out (KO) mice, reduced self-administration/reward in mice that do not express α4, α6, or β2 subunits, enhanced responsiveness to nicotine via increased activation or overexpression of the α4 subunit, as well as reduced nicotine-induced VTA DAergic cell firing in α5 KO mice (Marubio et al. 2003; Ngolab et al. 2015; Picciotto et al. 1998; Pons et al. 2008; Tapper et al. 2004; Morel et al. 2014). Studies with ethanol have also shown that activation of DAergic neurons in the posterior VTA is partially mediated by nAChRs, presumably leading to release of DA in the NAc (Hendrickson et al. 2010; Liu et al. 2013a; Zhao-Shea et al. 2011). Pharmacological studies have shown that α-conotoxin MII, which is a selective antagonist at α6, β3, and α3/β2 containing nAChRs, significantly decreases ethanol-mediated DA release (Jerlhag et al. 2006). When administered locally into the VTA, α-conotoxin MII also reduced self-administration and operant responding for ethanol and inhibited DA release in the NAc of rats (Kuzmin et al. 2009; Larsson et al. 2004). Furthermore, genetic manipulations have identified that nAChRs containing α4 and α6, also contribute to the rewarding properties of ethanol (Liu et al. 2013a, b; Guildford et al. 2016; Powers et al. 2013).

Direct infusion of ethanol into the NAc can increase DA to a level that is similar in magnitude to systemic administration (Ericson et al. 2003). This effect may be due, in part, to direct ethanol-induced modulation of nAChRs on DAergic terminals

in the NAc. Within the NAc, large cholinergic neurons (Phelps et al. 1985) release acetylcholine which can activate nAChRs on presynaptic dopaminergic terminals to augment DA activity (Zhou et al. 2002). Previous studies have also observed a dense level of cholinergic fibers between the border of the NAc core and shell (Meredith et al. 1989). This area has been proposed as a potential site for nAChR-mediated DA responses to ethanol within the NAc. This is based on work that has observed increased DA levels within this region during ethanol self-administration (Howard et al. 2009). Additionally, infusion of α4/β2 nAChR partial agonist varenicline into the NAc core-shell border reduces voluntary ethanol consumption and increases DA release along with other nAChR antagonists, in a manner that is cell activity dependent (Feduccia et al. 2014). Nicotine can also have direct effects at DAergic terminals within the NAc (Fu et al. 2000; Nisell et al. 1994). Interestingly, this effect is thought to occur within the NAc shell (Kleijn et al. 2011), and appears to involve activation of α7 subunit-containing nAChRs (Fu et al. 2000) which is in contrast to ethanol.

2.3 Molecular, Neuronal, and Behavioral Effects of Acute Ethanol and Nicotine Consumption

The effects on brain circuitry resulting from the combined use of alcohol and nicotine have been less well studied than the effects mediated by consumption of each drug alone. However, recent studies have uncovered potential mechanisms that may contribute to the frequent co-use of alcohol and nicotine. Behavioral studies have shown that acute nicotine decreases the consumption of ethanol in self-administration and operant conditioning paradigms (Hauser et al. 2012; Le et al. 2000; Sharpe and Samson 2002; Tritto et al. 2001). Based on these results, it has been suggested that when nicotine is initially combined with ethanol, it acts as an additional reinforcer, which might reduce the amount of ethanol required for reward satiety (Sharpe and Samson 2002). This has been supported by work showing that systemic administration of ethanol and nicotine had an additive effect on the release of DA in the NAc (Tizabi et al. 2007). Additionally, it was revealed that nicotine infusion into the VTA combined with systemic ethanol potentiated accumbal DA release (Tizabi et al. 2002). A more recent study suggests that the additive effect of ethanol and nicotine on accumbal DA levels may involve nAChRs containing the β2 and β4 subunits (Tolu et al. 2017).

It has also been suggested that ethanol and nicotine co-use reduces the aversive side effects associated with consumption of each drug alone. This is supported by evidence revealing that acute nicotine administration significantly reduces ethanol-induced motor impairment (Dar et al. 1993, 1994) and cognitive deficits (Gould et al. 2001; Gould and Lommock 2003), suggesting that opposing effects could maintain the anxiolytic properties of ethanol while reducing cognitive impairment (Perkins 1997). Recent studies have shown that neurons within the rostromedial tegmental nucleus are activated in response to the aversive properties of both ethanol and nicotine (Fowler and Kenny 2014; Glover et al. 2016; Tandon et al. 2017). Increased

activity of GABAergic rostromedial tegmental nucleus neurons mediated by nicotine is thought to involve activation of α7 subunit-containing nAChRs on presynaptic glutamatergic inputs that project from the lateral habenula (Lecca et al. 2011). Similarly, recent work suggests that neurons within the rostromedial tegmental nucleus and lateral habenula are activated in response to the aversive properties of ethanol (Glover et al. 2016). If ethanol can modulate the activity of nicotine at nAChRs within this circuitry, this could provide a possible explanation for how ethanol and nicotine co-use might augment the aversive side effects associated with the consumption of each drug individually.

Following repeated use, additional processes are thought to lead to the development of cross-tolerance (de Fiebre and Collins 1993), which increases the consumption of ethanol and nicotine compared to if the drugs were consumed individually (Zacny 1990). A possible explanation for this effect may involve changes in nicotine-induced nAChR activation and desensitization in the presence of ethanol. For example, although inhibiting α7 subunit-containing nAChRs does not affect ethanol consumption (Le et al. 2000) or block ethanol-induced DA release in the NAc (Ericson et al. 2003; Larsson et al. 2002), ethanol has been shown to inhibit α7 subunit-containing nAChRs in cultured cortical neurons by potentially enhancing desensitization of the receptor (Aistrup et al. 1999; Dopico and Lovinger 2009). Ethanol also reduces nicotine-induced glutamate release from laterodorsal tegmental nucleus neurons in a PKA-dependent manner (McDaid et al. 2016). Additionally, nAChRs containing α4 and β2 subunits which are rapidly desensitized by nicotine were found to have increased activity in the presence of ethanol and desensitizing concentrations of nicotine (Aistrup et al. 1999).

2.4 Neuronal and Molecular Effects of Nicotine That Induce Escalating Ethanol Consumption

Because the long-term effects of ethanol and nicotine co-use on the dopaminergic system at a molecular level remain unknown, it is not clear whether combined influences at nAChRs and other receptor subtypes may increase the susceptibility and/or expedite the development of dependence compared to consumption of each drug alone. It is possible that molecular consequences of ethanol and nicotine co-use could increase the likelihood and accelerate adaptations associated with dependence. For example, during the development of alcohol dependency, repeated use often causes an escalating pattern of binge-like intake over time (Koob and Volkow 2010). A recent study showed that ethanol and nicotine co-exposure in rats increased the rate at which ethanol consumption escalated compared to ethanol exposure alone (Leao et al. 2015). Moreover, this effect increased compulsive drinking, accelerated the transition to dependence, and was blocked by the nAChR antagonist mecamylamine (Leao et al. 2015). Results from this study also revealed that nicotine-induced increases in ethanol intake involved increased neuronal activation in the dorsomedial PFC, the NAc core, and the central nucleus of the amygdala (CeA). In addition to interactions on the mesolimbic system, the opposing roles of ethanol and nicotine on other molecular targets including glutamate transporters (Flatscher-Bader et al. 2008) and choline

acetyltransferase (Hernandez and Terry 2005; Jamal et al. 2009) could also contribute to nicotine-induced escalation of ethanol intake. Additional evidence suggests that metabolic changes may also be involved in subsequent increases in consumption during ethanol and nicotine co-use (Collins et al. 1996; Schoedel and Tyndale 2003).

2.5 Genetic Variations Implicated in Ethanol and Nicotine Consumption

Recent genomic studies have provided evidence to suggest that variation in genes may predispose and/or contribute to the development of drug addiction in certain individuals. For example, variations in genes that contribute to the reinforcing properties of ethanol and nicotine, including genes encoding nAChR subunits and alcohol metabolizing enzymes, are implicated in the development of alcohol and nicotine addiction [for review, see (Tawa et al. 2016; Yang and Li 2016)]. Of note, polymorphisms in the nAChR subunit genes that are clustered in the genome, CHRNA3-CHRNA5-CHRNB4 (encoding α3, α5, and β4 subunits), as well as CHRNB3-CHRNA6 (encoding β3 and α6 subunits), have been associated with nicotine dependence susceptibility (Bierut et al. 2008; Thorgeirsson et al. 2008, 2010). Polymorphisms in CHRNA3-CHRNA5-CHRNB4 have also been linked to age of initiation for both nicotine and alcohol (Schlaepfer et al. 2008). In addition, unique polymorphisms in CHRNA3-CHRNA5 and rare variants of CHRNB3 and CHRNA3 have been associated with risk for alcohol dependence (Haller et al. 2014; Wang et al. 2009). However, while the high incidence of ethanol and nicotine co-use suggests that common genetic influences may exist, to date, only two genome-wide association studies have investigated variations involved in alcohol and nicotine co-dependence (Lind et al. 2010; Zuo et al. 2013). Findings from these studies revealed associations between alcohol and nicotine co-addiction and single nucleotide polymorphisms located near or within the microtubule affinity regulating kinase 1 gene, the DEAD-box helicase 6 gene, the NALCN channel complex subunit gene and between the importin 11 and 5-hydroxytryptamine receptor 1A genes. Moreover, a recent study looking at DNA methylation, which is an epigenetic mechanism that can alter gene expression without changing the DNA sequence, found significant variations in genes of several neurotransmitter systems including serotonin, DA, GABA, glutamate, and opioids (Xu et al. 2017). These results are likely to represent a small percentage of the genetic influences contributing to the co-use of ethanol and nicotine. Therefore, additional genomic investigations are likely to identify other genetic variations involved in the co-occurrence of alcohol and nicotine use.

3 Neural Circuitry Involved in the Development of Alcohol and Nicotine Dependence

Prolonged use of ethanol and nicotine elicits unique adaptations that are lacking following short-term use. These changes are consolidated in various neurochemical pathways and play a critical role in the development of dependence. In some cases,

these adaptations are thought to occur as homeostatic responses that initially compensate for changes initially occurring in the presence of high ethanol and nicotine concentrations. The development of alcohol and/or nicotine dependence is initiated when tolerance leads to more frequent drug intake and hedonic dysfunction (Koob and Le Moal 1997). Over time, this can lead to an inability to self-limit intake despite negative consequences and cause the emergence of negative emotional states during withdrawal (Koob and Volkow 2010), that facilitate craving and relapse during abstinence.

3.1 Adaptations in Brain Reward Circuitry

A number of recent studies have uncovered several potential mechanisms that are involved in the development of nicotine dependence. For example, differences in the rate of desensitization are thought to play a pivotal role in the sustained nicotine-induced activation of DAergic neurons in the VTA. Previous studies have reported that, in the VTA, rapid desensitization of α4/β2 nAChRs within local GABAergic neurons and GABAergic projections combined with the activation of α7 containing nAChRs on glutamatergic terminals which do not desensitize as quickly, leads to an overall increase in excitatory drive onto DAergic neurons (Mansvelder et al. 2003; Mansvelder and McGehee 2000). When this effect is coupled with post-synaptic activation of nAChRs by nicotine, the subsequent depolarization of VTA DAergic neurons produces long-term potentiation (LTP) of glutamatergic transmission through NMDA and non-NMDA receptors (Mansvelder and McGehee 2000). These effects can increase the burst firing of DAergic VTA neurons and facilitate sustained DA release in the NAc (Mameli-Engvall et al. 2006; Schilstrom et al. 2003). Additionally, cholinergic modulation of GABAergic interneurons in the VTA has also been shown to play an important role in the burst activity of DAergic neurons (Tolu et al. 2013).

The chronic activation and desensitization of nAChRs induced by prolonged nicotine use is thought to facilitate mechanisms that cause an upregulation of receptor expression in the VTA and NAc. In particular, upregulation of α4/β2 nAChRs has been demonstrated following chronic nicotine treatment (Flores et al. 1992). The upregulation of nAChRs in response to chronic nicotine is thought to represent an underlying molecular mechanism that, in part, facilitates tolerance by desensitizing the mesolimbic system. One proposed mechanism includes upregulation of α4 subunit-containing nAChRs on GABAergic neurons in the VTA, which in chronically nicotine treated mice increases their basal activity (Nashmi et al. 2007), thereby enhancing inhibition and reducing the activity of VTA DAergic neurons (Nashmi et al. 2007). Neuronal adaptations such as these could affect the threshold required for reward satiety and increase the propensity to consume alcohol. Conversely, high ethanol concentrations may, to some extent, re-sensitize the rewarding properties of nicotine by increasing the activity of GABAergic inputs to GABAergic neurons in the VTA. This is supported by animal data showing that chronic nicotine administration increases ethanol self-administration (Clark et al. 2001; Le et al. 2003; Potthoff et al. 1983; Smith et al. 1999), and the involvement of nAChRs in the locomotor stimulant

response to ethanol (Blomqvist et al. 1992). Additional work has shown that chronic nicotine can induce an upregulation of nAChRs that contain certain combinations of nAChR receptor subunits. Examples include α3 and α4 subunit-containing nAChRs that also contain a β2 subunit (Sallette et al. 2004; Wang et al. 1998), and α6/β2 subunit-containing nAChRs that also contain a β3 subunit (Tumkosit et al. 2006). A number of mechanisms have been proposed to explain nicotine-induced upregulation of nAChRs including a response to receptor desensitization, which occurs during sustained nicotine exposure (Benowitz 2008). Additionally, it has been shown that nicotine can act as a "chaperone" which expedites the transport of nAChR subunits to the endoplasmic reticulum and facilitates the passage and insertion of assembled nAChRs to the plasma membrane (Henderson et al. 2014; Kuryatov et al. 2005; Srinivasan et al. 2011).

3.2 Molecular and Synaptic Effects of Ethanol and Nicotine on Brain Circuitry Involved in Reinforcement-mediated Learning and Memory Underlying the Development of Addiction

An important aspect of addiction is the development of associative memories that pair drug use with specific environments or cues. The formation of drug-context associations that relate drug-predictive environments or cues to drug reward can have a strong influence on behavior by contributing to drug craving and relapse during abstinence (Crombag et al. 2008). Recent studies implementing conditioned place preference, where nicotine administration is paired with a specific environment or context have shown that connections between the tegmental pedunculopontine nucleus and the VTA are involved in these associations (Laviolette et al. 2002). More recent studies have shown that nAChRs containing the α4, α6, and β2 subunits (Ngolab et al. 2015; Sanjakdar et al. 2015; Walters et al. 2006) in addition to other receptor subtypes including $GABA_B$, cannabinoid, and α1-adrenoceptors also play a role in contextual memories involving nicotine administration (Forget et al. 2009; Hashemizadeh et al. 2014; Navarrete et al. 2013).

Ethanol can also induce place preference in rodents with previous studies showing that projections from the amygdala to NAc are involved during memory acquisition (Gremel and Cunningham 2008) and retrieval (Theberge et al. 2010). Studies have also implicated the involvement of DAergic transmission during this process as local infusion of DA receptor antagonists into the NAc reduces the formation of ethanol conditioned place preference (Walker and Ettenberg 2007; Young et al. 2014). Following consolidation of the paired ethanol-context memory, GABAergic activity in the VTA, DAergic neuron innervation in the amygdala, glutamatergic inputs from the amygdala to the NAc and inactivation of the bed nucleus of the stria terminalis (BNST) contribute to and can affect memory expression (Bechtholt and Cunningham 2005; Gremel and Cunningham 2009, 2010; Pina et al. 2015).

It is conceivable that upregulation of nAChRs in addition to other adaptations resulting from repeated ethanol and nicotine co-use may enhance craving when drug associated environments or cues are encountered during abstinence. Animal studies have shown that

when a conditioned stimulus (which acts as drug-predictive cue) is paired with ethanol, presentation of the cue is sufficient to induce accumbal DA release (Lof et al. 2007). Moreover, DA receptor antagonists decrease cue-induced responding for nicotine (Cohen et al. 2005; Khaled et al. 2010; Liu et al. 2010). Studies have also highlighted the involvement of nAChRs in cue-induced reinforcement for ethanol and nicotine. For example, local VTA infusion of the non-selective nAChR antagonist mecamylamine blocked the DA response following cue presentation and reduced conditioned-responding for ethanol (Lof et al. 2007). For nicotine, the selective α7 antagonist, methyllycaconitine, was found to decrease cue-induced reinstatement (Liu 2014). The involvement of glutamatergic synaptic transmission is also thought to play an important role in the development of paired nicotine-cue associations with previous reports identifying increased extracellular glutamate, changes in glutamate receptor expression, and increased excitatory post-synaptic currents following reinstatement of nicotine-seeking behavior by conditioned cues (Dravolina et al. 2007; Gipson et al. 2013; Liechti et al. 2007). The combined effects of nicotine at nAChRs and glutamate receptors are thought to potentiate synaptic responses that underlie learning processes and memory formation. Interestingly, previous studies have shown that ethanol and nicotine co-use can change the response to paired drug–cue associations. For example, in mice, it has been shown that ethanol in excess of 0.5 mg/kg in a combined ethanol and nicotine mixture can "overshadow" the salience of a cue previously paired with nicotine (Ford et al. 2012). This effect is thought to involve an ethanol-induced, antagonist-like effect at NMDA receptors (Ford et al. 2013). This is in contrast to the effect of nicotine in the presence of low dose ethanol (<0.5 mg kg) which can potentiate the discriminative effects of ethanol (Ford et al. 2012; Kouri et al. 2004). Importantly, these studies highlight the actions of ethanol and nicotine at nAChRs and other receptor targets that contribute to paired drug–cue associations and reinstate drug-seeking following the presentation of environmental and contextual cues. Studies are beginning to uncover the cellular and synaptic mechanisms underlying these behavioral responses, which are thought to contribute to craving and facilitate relapse abstinence.

The activation of nAChRs by nicotine can induce synaptic plasticity and long-lasting signalling effects in a number of brain regions involved in the formation and consolidation of associative memories including the PFC, amygdala, and hippocampus. For example, in the PFC, nicotine-induced activation of α7 and β2 subunit-containing nAChRs increases DA levels (Livingstone et al. 2009; Marshall et al. 1997) and DA binding to D_1 and D_2 receptors can initiate long-term depression (LTD) when paired with post-synaptic membrane depolarization (Law-Tho et al. 1995; Otani et al. 1998). In addition, it has been shown that spike-timing dependent potentiation of layer V pyramidal neurons in the PFC is reduced by nicotine via activation of nAChRs which changes glutamate receptor compositions within glutamatergic terminals, and activates low-threshold spiking GABAergic interneurons which combine to reduce dendritic calcium signalling following action potential propagation (Couey et al. 2007). This effect may improve the signal to noise ratio during PFC mediated information processing and enhance cognitive performance (Couey et al. 2007).

Chronic nicotine consumption also enhances long-term potentiation (LTP) following stimulation of cortical inputs to the lateral amygdala (Huang et al. 2008). This effect was observed in mice that consumed nicotine in water for 7 days. The

nicotine-induced LTP persisted for 72 h following the last drinking session was found to involve α7 and β2 subunit-containing nAChRs and was NMDAR dependent (Huang et al. 2008). This effect was partially attributed to reduced GABergic activity mediated through nAChRs, however previous studies have also shown that nicotine enhances glutamatergic transmission in the basolateral amygdala (BLA) and potentiates excitatory responses from cortical inputs (Jiang and Role 2008).

In the hippocampus, high expression levels of α7 and α4/β2 subunit-containing nAChRs have been found on GABAergic interneurons and pyramidal cells with synaptic plasticity being reported via both excitatory and inhibitory mechanisms. In the CA1 region, nicotine can disinhibit pyramidal neuron activity through GABAergic interneurons and reduce evoked GABA release through α7 and non-α7 nAChRs, respectively (Fujii et al. 2000; Ji and Dani 2000; Yamazaki et al. 2005). Additionally, activation of α7 nAChRs on presynaptic glutamatergic terminals by nicotine can increase excitatory synaptic currents and glutamate release onto CA1 pyramidal neurons (Gray et al. 1996; Radcliffe and Dani 1998). The induction of LTP by nicotine in the dentate gyrus was found to involve a mechanism that required activation of α7 nAChRs and metabotropic glutamate receptors, in addition to the utilization of intracellular calcium stores through activated L-type calcium and ryanodine channels (Welsby et al. 2006). A mechanism of nicotine-induced LTP through α4/β2 containing nAChRs has also been demonstrated (Matsuyama and Matsumoto 2003) and is thought to rely on DAergic signalling from the midbrain (Tang and Dani 2009).

The mechanisms of synaptic plasticity described for ethanol are for the most part distinct from those of nicotine and have been proposed to result in an overall state that involves increased glutamatergic and reduced GABAergic activity. It is hypothesized that these mechanisms act as adaptive homeostatic responses to compensate for the initial effects of acute ethanol consumption. Acute ethanol can modulate the activity of several receptor subtypes including the enhancement of $GABA_A$ (Lobo and Harris 2008), glycine (McCool et al. 2003), and $5HT_3$ (Lovinger and White 1991) receptors and inhibition of NMDA (Kuner et al. 1993; Lovinger et al. 1989; Masood et al. 1994), AMPA (Lovinger 1993; Moykkynen et al. 2003) and kainate receptors (Valenzuela et al. 1998). Ethanol can also indirectly inhibit LTP and LTD in certain brain regions including the hippocampus (Zorumski et al. 2014) and the dorsal striatum (Clarke and Adermark 2010; Yin et al. 2007). Following repeated and/or chronic exposure to ethanol, additional responses can induce molecular and neurochemical changes that can have long-lasting effects on synaptic activity. For example, in VTA neurons of rats given intermittent access to ethanol for 5–7 weeks, an increase in AMPA-mediated post-synaptic currents and basal glutamate release was observed (Stuber et al. 2008). Chronic ethanol administration can also facilitate induction of LTP via NMDA-mediated post-synaptic currents on VTA DAergic neurons by enhancing 1,4,5-trisphosphate-mediated intracellular calcium release (Bernier et al. 2011).

The amygdala is another prominent site of action for ethanol-induced plasticity. Chronic ethanol administration causes an increase in AMPA-mediated post-synaptic currents in the BLA (Läck et al. 2007) and sensitizes NMDA receptors to ethanol in the CeA (Roberto et al. 2004b). In ethanol-dependent rats, increased GABAergic

activity is observed in CeA neurons in response to ethanol (Roberto et al. 2004a) and activation of $GABA_A$ receptors in the amygdala reduces ethanol-self administration (Roberts et al. 1996). Although further work is required to uncover mechanisms underlying changes in glutamatergic and GABAergic receptor function following long-term ethanol exposure, studies suggest that these changes could, in part, result from altered subunit expression and receptor composition (Floyd et al. 2003; Kash et al. 2009). This is supported by recent studies that have identified differences in expression of $GABA_A$ and glutamate receptor subunits in post-mortem analysis of human brains from control and alcohol-dependent individuals (Bhandage et al. 2014; Jin et al. 2011, 2014a, b).

These results suggest that repeated ethanol and nicotine consumption can have significant effects on brain circuitry implicated in the development of addiction. The long-lasting molecular and synaptic effects of combined ethanol and nicotine co-use on circuits involved in learning and memory, drug-reinforcement, and drug-reinstatement appear to have a strong influence on drug-seeking behavior and cause a persistent and increased susceptibility to relapse during abstinence. Although the effects of combined ethanol and nicotine co-use on mechanisms of synaptic plasticity remain largely unknown, future research may help to determine if mechanisms of plasticity have an additive affect that contribute to the high incidence of ethanol and nicotine co-use. Studies may also help to identify novel mechanisms that lead to more frequent consumption over time and establish whether ethanol and nicotine co-use accelerates the development of behavioral symptoms associated with dependence.

4 The Development of Ethanol and Nicotine Withdrawal

A key component of the addiction cycle is the development of withdrawal symptoms during abstinence. During withdrawal, severe negative emotions including dysphoria, anxiety, and stress increase craving and motivation for drug-taking and can lead to an ability to self-limit intake despite negative consequences (Koob and Volkow 2010). Withdrawal states associated with drug-dependence develop following extended periods of drug use and are thought to result from the consolidation of adaptations in brain circuits that control behavioral responses to negative emotional states. The effects of alcohol and nicotine in brain circuitry implicated in withdrawal are shown in Fig. 2.

4.1 Interactions of Ethanol and Nicotine on Brain Circuitry Involved in Withdrawal

The development of nicotine withdrawal is thought to involve an upregulation of nAChRs, which occurs following long-term exposure (Paolini and De Biasi 2011; Turner et al. 2011). Studies also suggest that chronic nicotine use can lead to a reduction in the activity of the mesolimbic pathway during withdrawal. For example, chronic nicotine infusion can cause a reduction in DA release from tonic firing of

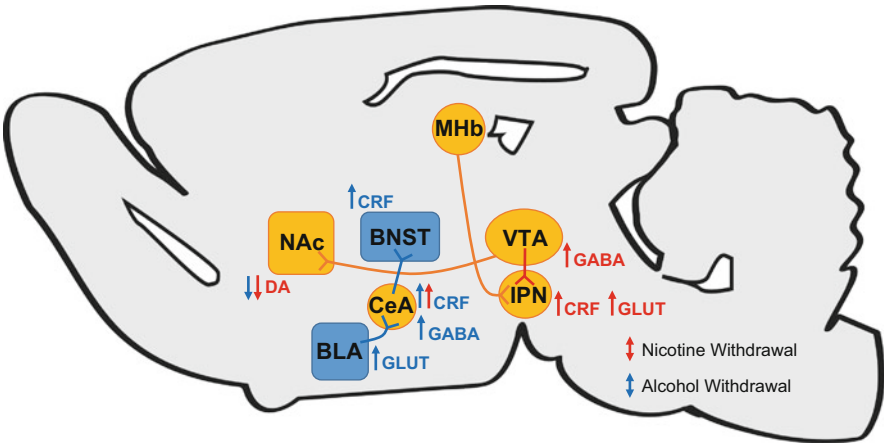

Fig. 2 The effects of ethanol and nicotine in brain circuitry involved in withdrawal. Alcohol (*blue*) and nicotine (*red*) target distinct and overlapping brain circuits (*orange*) that are involved in the development of withdrawal. Studies have shown that physical and anxiety-like symptoms of nicotine withdrawal involve glutamatergic and cholinergic input (*orange*) from the medial habenula (MHb, *orange*) to the interpeduncular nucleus (IPN, *orange*). Also implicated in nicotine withdrawal-induced anxiety is corticotrophin releasing factor (CRF, *red*) signalling within the interpeduncular intermediate subregion of the IPN, which may result from input of neighboring CRF-containing neurons in the ventral tegmental area (VTA, *red*). Another mechanism that may promote withdrawal-like symptoms is chronic nicotine-induced increases in the basal activity of VTA GABAergic neurons, greater inhibition of DAergic VTA neurons and reduced basal DA levels in the nucleus accumbens (NAc, *red*). During ethanol withdrawal, increased CRF levels in the central nucleus of the amygdala (CeA, *blue*) and the bed nucleus of the stria terminalis (BNST, *blue*) have been observed. In ethanol-dependent rats, increased glutamate (GLUT, *blue*) in the basolateral amygdala (BLA, *blue*) and the CeA is also found during withdrawal. Recently, activation of nAChRs within the MHb (*orange*) and IPN (*orange*) was shown to promote physical symptoms of alcohol withdrawal. These results offer the possibility that the combined effects of ethanol and nicotine within these pathways may cause more severe and prolong symptoms of withdrawal compared to individual consumption of each drug

DAergic VTA neurons (Grieder et al. 2012). This effect is also seen following infusion of nAChR antagonists into the VTA of rats chronically treated with nicotine (Carboni et al. 2000; Hildebrand et al. 1998; Rada et al. 2001). This suggests that following chronic nicotine exposure, sustained activation of nAChRs may play a role in inhibiting DAergic VTA neurons and reducing basal DA levels in the NAc. Furthermore, this effect appears to be enhanced following acute withdrawal from nicotine and is reversed following re-exposure (Zhang et al. 2012). Although studies show that as plasma levels of nicotine increase in response to chronic exposure, the DA effect is diminished (Balfour 2009), the initial increase in extracellular DA elicited by nicotine may provide a modest hedonic effect that may help to prolong abstinence from alcohol in alcoholic smokers.

During prolonged abstinence from nicotine, evidence suggests that a persistent upregulation and reduced desensitization of nAChRs may lead to a hyperactive cholinergic system, which facilitates affective and somatic symptoms of nicotine withdrawal. In

studies implementing pharmacological interventions and nAChR receptor subunit knock-out mice, results have identified a role for α2 (Lotfipour et al. 2013), α3 (Jackson et al. 2013), α5 (Salas et al. 2009), α6 (Jackson et al. 2009), β2 (Jackson et al. 2008), and β4 (Salas et al. 2004) nAChR receptor subunits in the expression of nicotine withdrawal symptoms. These receptor subunits exhibit high expression levels in the VTA [α5, (Salas et al. 2003a); α7, (Zhao-Shea et al. 2011)] and the medial habenula (MHb) to interpeduncular nucleus (IPN) [α2, (Lotfipour et al. 2013); α3, (Grady et al. 2009; Shih PY et al. 2014); α5, (Salas et al. 2003a); β4, (Salas et al. 2003b; Shih PY et al. 2014)] pathway. The MhB-IPN circuit contains efferent outputs from the MHb that release glutamate, acetylcholine, and substance P in the IPN (Zhao-Shea et al. 2013). This circuit plays a critical role in nicotine withdrawal. Pharmacological studies have demonstrated that injection of the nAChR antagonist mecamylamine into the Mhb or IPN precipitates affective and somatic symptoms of nicotine withdrawal (Salas et al. 2009; Zhao-Shea et al. 2013). The induction of withdrawal symptoms was observed following light-stimulated activation of GABAergic neurons in the IPN (Zhao-Shea et al. 2013) of mice expressing channelrhodopsin in GAD2-expressing neurons. Furthermore, increased β4 subunit expression was found in somatostatin expressing IPN neurons of nicotine-dependent mice and blockade of β4 containing nAChRs with SR16584 induced withdrawal-like symptoms in nicotine-naïve animals (Zhao-Shea et al. 2013). Glutamatergic and cholinergic input from the Mhb to the IPN was also found to contribute to symptoms of nicotine withdrawal (Zhao-Shea et al. 2013; Pang et al. 2016).

Recent evidence indicates that the MHb-IPN circuit is also involved in ethanol withdrawal. Following a chronic ethanol treatment, blockade of nAChRs in the MHb or IPN with mecamylamine induced withdrawal-like symptoms in mice (Perez et al. 2015). This study also found that the length and severity of withdrawal was significantly increased following ethanol and nicotine co-use compared to each drug individually (Perez et al. 2015). The dual effects of ethanol and nicotine on the MHb-IPN pathway make it an important candidate for future studies aimed at investigating whether molecular and neuronal adaptations in this circuit contribute to the high incidence of ethanol and nicotine co-use and increase susceptibility for relapse. The identification of neurochemical changes within this pathway such as the absence of neuropeptide Y in the MHb of alcohol-preferring rats (Hwang et al. 2004) could be targeted in order to develop improved pharmacotherapies for the management of alcohol and nicotine co-use.

Another possible interaction between ethanol and nicotine during withdrawal may involve dual effects on corticotrophin releasing factor (CRF) signalling in extrahypothalamic brain areas including the extended amygdala. During ethanol withdrawal, increased levels of CRF are observed in the CeA and BNST (Merlo Pich et al. 1995; Olive et al. 2002). Furthermore, CRF_1 receptor antagonism in ethanol-dependent and ethanol-withdrawn animals produces a robust decrease in consumption (Funk et al. 2007; Roberto et al. 2010) and reduces withdrawal-induced anxiety (Rassnick et al. 1993). The effects of CRF on ethanol consumption appear to involve an interaction with GABAergic transmission. Specifically, ethanol-induced enhancement of GABAergic activity is mediated via CRF_1 receptors (Nie et al. 2004) and CRF_1 receptor antagonism in the CeA reverses increases in GABA release

induced by ethanol dependence (Roberto et al. 2010). Additionally, the activity of a subpopulation of CRF_1 receptor containing neurons within CeA is enhanced by ethanol and ethanol-mediated increases in CeA projections to the BNST were also observed (Herman et al. 2013).

CRF signalling within the CeA has also been implicated in nicotine withdrawal. The induction of withdrawal with mecamylamine in chronically-nicotine-treated mice causes an increase in CRF levels within the CeA (George et al. 2007). Blockade of CRF_1 receptors within the CeA also reduces mecamylamine-precipitated and abstinence-induced anxiety (Cohen et al. 2015; George et al. 2007). Recent studies have also identified that CRF activity in the IPN contributes to nicotine withdrawal-induced anxiety (Zhao-Shea et al. 2015). This effect was mediated via CRF_1 receptors within the interpeduncular intermediate subregion of the IPN (Zhao-Shea et al. 2015) and may reply upon neighboring CRF-containing neurons in the VTA which project to the IPN (Grieder et al. 2014). Studies also suggest that interactions between the CRF and cholinergic systems could affect processes underlying learning and memory (Warnock et al. 2006). These results suggest that interactions between ethanol and nicotine on CRF signalling could potentially have effects that prolong or increase the severity of stress- and anxiety-like responses during withdrawal. Because CRF signalling in other brain areas including the dorsal raphe nucleus and mPFC also play a role in drug-reinforcement and drug-seeking behaviors (Zorrilla et al. 2014), more detailed studies aimed at uncovering the long-term effects of ethanol and nicotine co-use on the CRF system could help identify novel neuronal mechanisms involved in the development of alcohol and nicotine co-dependence.

5 Summary and Conclusions

Despite recent advancements in our understanding of brain circuitry and the molecular and neuronal mechanisms contributing to the development of alcohol and nicotine addiction, there remains a critical need for future research aimed at understanding the long-term effects of alcohol and nicotine co-use, in order to identify improved treatment strategies for the management of alcohol and nicotine co-dependence. A number of studies have uncovered important interactions between alcohol and nicotine that appear to contribute to the high incidence of their co-use. Furthermore, studies suggest that nicotine can facilitate an escalation in alcohol intake and produce additional responses compared to alcohol consumption alone, that reinforce drug-seeking behavior and facilitate craving and relapse during abstinence. However, due to lack of long-term studies, the molecular and neuronal adaptations caused by prolonged alcohol and nicotine co-use remain to be elucidated. In addition, the implication that extended alcohol and nicotine consumption could prolong symptoms of withdrawal and increase susceptibility to relapse compared to consumption of each drug individually also requires further investigation and may help to identify improved pharmacotherapeutics for the management of use disorders associated with alcohol and nicotine co-use.

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Cross-Species Alterations in Synaptic Dopamine Regulation After Chronic Alcohol Exposure

Cody A. Siciliano, Anushree N. Karkhanis, Katherine M. Holleran, James R. Melchior, and Sara R. Jones

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Abstract

Alcohol use disorders are a leading public health concern, engendering enormous costs in terms of both economic loss and human suffering. These disorders are characterized by compulsive and excessive alcohol use, as well as negative affect and alcohol craving during abstinence. Extensive research has implicated the dopamine system in both the acute pharmacological effects of alcohol and the

C. A. Siciliano
The Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology (MIT), Cambridge, MA, USA

A. N. Karkhanis · K. M. Holleran · J. R. Melchior · S. R. Jones (✉)
Department of Physiology and Pharmacology, Wake Forest School of Medicine, Winston-Salem, NC, USA
e-mail: srjones@wakehealth.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_106

symptomology of alcohol use disorders that develop after extended alcohol use. Preclinical research has shed light on many mechanisms by which chronic alcohol exposure dysregulates the dopamine system. However, many of the findings are inconsistent across experimental parameters such as alcohol exposure length, route of administration, and model organism. We propose that the dopaminergic alterations driving the core symptomology of alcohol use disorders are likely to be relatively stable across experimental settings. Recent work has been aimed at using multiple model organisms (mouse, rat, monkey) across various alcohol exposure procedures to search for commonalities. Here, we review recent advances in our understanding of the effects of chronic alcohol use on the dopamine system by highlighting findings that are consistent across experimental setting and species.

Keywords
Alcohol · Autoreceptors · Cross-species · Dopamine · Kappa Opioid receptors · Monkey · Mouse · Nonhuman primate · Rat · Uptake

1 Introduction

1.1 Alcohol Use Disorder

Alcohol use disorder (AUD) is a medical diagnosis describing a cluster of symptoms characterized by compulsive and excessive alcohol use despite negative health and social outcomes of drinking (Dziegielewski 2010; Hagman and Cohn 2011; O'Brien 2011). AUD is a leading public health concern, affecting roughly 14% of the adult population in the United States (Grant et al. 2015). The prognosis for AUD is often poor, and it is estimated that alcohol use leads to around 100,000 deaths per year in the United States alone, and 4% of deaths worldwide, making it the third leading preventable cause of death (Mokdad et al. 2004; Rehm et al. 2009). Beyond mental and physical deterioration, alcoholism and the larger spectrum of AUD also negatively affect civil and social responsibilities and interpersonal relationships. AUDs are associated with higher incidences of stress, anxiety, depression, and other mood disorders (Hasin et al. 2007), which may contribute to the maintenance of alcohol use as an anxiolytic (Blaine and Sinha 2017). In addition, AUD is a chronically relapsing disease (Dawson et al. 2007; Pickens et al. 1985). Given the severe negative outcomes of this prevalent brain disorder, the alcohol research field has focused its efforts on understanding the neurobiology of AUD in an effort to develop effective therapeutic strategies. Here we will review recent advances in understanding the mechanisms by which alcohol exposure affects dopamine signaling to produce aberrant behaviors seen in AUD.

1.2 The Role of Dopamine in Alcohol Use Disorder Symptomology

Many drugs of abuse exert their subjective effects (euphoria, or "high"), in part, via actions on the mesolimbic dopamine system (Di Chiara and Imperato 1988; Siciliano et al. 2015b; Volkow et al. 1997). Alcohol, like many abused substances, increases extracellular dopamine concentrations in the ventral striatum, an area known to be involved in reward and motivation (Humphries and Prescott 2010; Imperato and Di Chiara 1986). Acutely, alcohol increases dopamine signaling via directly targeting ion channels expressed on dopamine neurons, which alter the currents that shape cellular communication, and result in increased firing of dopamine neurons in the ventral tegmental area (VTA), leading to increased dopamine release downstream in the ventral striatum (Brodie 2002; Brodie et al. 1990, 1999; Nimitvilai et al. 2016). Alcohol enhances hyperpolarization-activated depolarizing cation currents (I_h), which increase intrinsic activity in dopamine neurons (Okamoto et al. 2006). In addition, alcohol modulates various potassium channel (K^+)-mediated aspects of hyperpolarization (Appel et al. 2003). For example, alcohol has been shown to modulate large-conductance potassium (BK) channels (Chu et al. 1998), G-protein-coupled inwardly rectifying K^+ channels (GIRK) (Aryal et al. 2009), and K^+-channel mediated M currents (Koyama et al. 2007). Additionally, alcohol has been shown to alter L-type Ca^{2+} channels (Hendricson et al. 2003), which may alter dopamine cell firing. While it is important to note that alcohol acutely modulates dopaminergic activity, this review will focus on the adaptions to the dopamine system induced by chronic alcohol use. Further, this review will focus on receptor and circuit level analyses; for more in-depth discussions of the molecular mechanisms underlying these alterations we point the reader towards several other helpful reviews (Morikawa and Morrisett 2010; Abrahao et al. 2017; Morisot and Ron 2017; Ron and Barak 2016).

Changes in dopamine signaling are particularly important in the context of alcohol abuse, as the dopamine system plays a key role in mediating adaptive decision-making. Dopamine neurons in the VTA are required for adaptive encoding of reward-predictive cues, which allows organisms to successfully navigate complex environments and acquire rewards, such as food (Nicola 2010; Phillips et al. 2003; Schultz et al. 1997). Dynamic activity of dopamine neurons, and dopamine release downstream in areas such as the striatum, are critical to both initial learning of reinforcement contingencies and updating the value of these contingencies as they shift over time (Cools et al. 2009; Schultz 2013). Continued alcohol use can, in some individuals, induce a maladaptive shift in contingency valuation such that the motivational saliency of alcohol is increased, leading to behaviors aimed at acquiring alcohol at the expense of more adaptive rewards. Because alcoholics show a decreased ability to make adaptive decisions – and instead continue maladaptive behavioral strategies such as alcohol seeking – the dopamine system has been implicated as a probable locus of these behavioral aberrations.

In humans, it is known that chronic alcohol use dysregulates the dopamine system. For example, alcohol abusers show greatly reduced dopamine signaling in

the ventral striatum (Diana et al. 1993; Martinez et al. 2005; Volkow et al. 1996, 2007). This reduced dopamine signaling is often referred to as a "hypodopaminergic state" and is also observed in other addictive disorders such as psychostimulant addiction (Melis et al. 2005). It is hypothesized that this low-dopamine state results in deficits in reward processing, which contributes to anhedonia during withdrawal from alcohol (Danjo et al. 2014; Schulteis et al. 1995). Because anhedonia, defined as a reduced ability to experience pleasure or reward, is thought to occur primarily in relation to non-alcohol stimuli, this may bias choices towards previously reinforced alcohol seeking behaviors over alternative options (Pierce et al. 1990; Rebec et al. 1997; Twining et al. 2015), resulting in continued and persistent alcohol use. Anhedonia, maladaptive decision-making, and alcohol seeking are sine qua non symptoms of AUD. The precise mechanisms by which alcohol disrupts dopamine system function are difficult to study in humans, and thus have been a major focus of preclinical alcohol abuse research.

1.3 Preclinical Models of Alcohol Abuse

A great deal of research into the neurobiological basis of AUD has been conducted in animal models, where hypotheses can be readily tested via direct measurements and manipulations of the receptors and circuits involved. Preclinical studies have overwhelmingly leveraged rodent (rat and mouse) and nonhuman primates as model organisms. Rodent and nonhuman primate models each offer specific advantages in exploring alcohol's effects on the brain. For example, rodents can be procured and bred quickly, and are thus more practical for studies which require brain tissue to be harvested (e.g., for protein analysis, or ex vivo slice preparations) or where high-risk, invasive methods are needed (e.g., intracranial implants). Further, there are a wide array of tools that have been developed for use in rodents, including genetic modifications, in vivo microscopy, neurotransmitter sensors, and tools for manipulating neural circuit activity (Boyden et al. 2005; Flusberg et al. 2008; Koller and Smithies 1992; Wightman 1988; Wightman et al. 1976). Many of these techniques have been adapted for, and implemented in, nonhuman primates; however, they are generally less developed and often pose greater challenges (Ariansen et al. 2012; Eldridge et al. 2016; Stauffer et al. 2016).

Nonhuman primate models offer many advantages over rodents, although invasive approaches are often not feasible. First, they are genetically much closer to humans, as macaque monkeys share 95% gene homology with humans while mice share only 75% homology (Church et al. 2009). This genetic similarity manifests itself in, among other things, a high degree of correspondence in neuroanatomical structures between monkeys and humans (Seress 2007). High levels of homology increase the likelihood that discoveries will generalize from nonhuman primates to humans, compared to lower organisms. The advantages of nonhuman primates' similarity to humans is even greater in studies of alcohol drinking, as the patterns of alcohol consumption in nonhuman primates are similar to humans (Grant et al. 2008; Majchrowicz and Mendelson 1970). While rodents metabolize alcohol at a

much faster rate than humans, nonhuman primates have similar alcohol metabolism as humans. Further, AUD is a chronic disorder which can take years to develop, and lasts a lifetime (Dawson et al. 2008). Not only is AUD long lasting, but there are also interactions between drinking and age/developmental periods. For example, age at first drink is strongly predictive of problematic drinking behaviors later in life (Dawson et al. 2008). The large disparity between the human and rodent life span can make examining the effects of alcohol exposure over long periods of time or during specific developmental periods challenging (Silberberg and Silberberg 1954). Macaques, which have been used most frequently for alcohol studies in the nonhuman primate literature, have a lifespan of 25–35 years in captivity, and thus are often more appropriate for longitudinal experimental questions (Tigges et al. 1988).

1.4 Importance of Consistent Cross-Species Results

Rodent and nonhuman primate models have provided valuable insight into the neurobiological and pharmacological basis of AUD; here, we posit that the most important insights from these literatures are the consistencies that can be observed across species. Searching for these consistencies is particularly important because the effects of chronic alcohol exposure in preclinical models is extremely sensitive to experimental parameters such as alcohol concentration, exposure length, route of administration, withdrawal length, strain of rodent or species of nonhuman primate, and many other variables that can have large impacts on the observed effects (Bonthius and West 1990; Budygin et al. 2003; Hwa et al. 2011; Kashem et al. 2012; Rimondini et al. 2003; Siciliano et al. 2016b, 2017). However, the core behavioral symptomology of AUD (excessive alcohol consumption, craving/seeking, and withdrawal behaviors) is relatively consistent across experimental parameters (Green and Grahame 2008; Le et al. 1998; Macey et al. 1996; Venniro et al. 2016), suggesting that neurochemical adaptations that are only observed under very specific experimental settings may not be driving the primary symptomology of AUD. Instead, it is likely that some of the alterations that are inconsistent across experimental setting may be "epiphenomenon" of the specific paradigm or model organism used. The large number of methodological differences across laboratories makes determining the source of any inconsistent effects difficult.

Comparing across species, strain, sex, and experimental parameters to search for consistent adaptations induced by chronic alcohol exposure is a powerful approach for filtering "noise" out of large sets of studies. Here, we will review commonalities and disparities in studies examining chronic alcohol-induced alterations to the dopamine system across multiple model organisms.

1.5 Alcohol Exposure Protocols Across Species

Each species utilized as a preclinical model of AUD described in this chapter provides unique assets to address and examine specific facets of alcoholism. The three model organisms discussed here (mouse, rat, and monkey) are distinct in terms of alcohol metabolism and intake pattern; thus, species-specific alcohol exposure protocols are often used. These protocols are described in the sections below and depicted in Fig. 1.

1.5.1 Chronic Intermittent Alcohol Exposure in Mice and Rats

Rodents (both mice and rats) will voluntarily consume alcohol under certain conditions; however, due to relatively low intakes and fast metabolism of alcohol, environmental or genetic manipulations are often required to produce high blood alcohol levels in these animals (Li et al. 1979; Penn et al. 1978; Rhodes et al. 2005, 2007). For example, selectively breeding animals with high alcohol intake has resulted in alcohol preferring strains of rats and mice (Penn et al. 1978). Environmental manipulations often include removing access to water or allowing alcohol access at specific times during the light cycle. Another approach to exposing animals to alcohol is a noncontingent method of alcohol administration in which rodents inhale tightly controlled levels of vaporized alcohol (for review see Gilpin et al. 2008). Because the levels are experimenter controlled (i.e., not dependent on the actions of the animal) this allows for titration of blood alcohol levels around a desired amount. Vapor alcohol exposure is often used to rapidly induced alcohol dependence by inducing very high blood alcohol levels ($\geq$200 mg/dL) for extended periods of time (Anderson et al. 2016a; Diaz et al. 2011; Rose et al. 2016). This blood alcohol level is approximately three times the legal limit for motor vehicle operation in the United States; it is important to achieve such high blood alcohol concentrations intermittently in order to drive dependence in rodents (Griffin et al. 2009; Rose et al. 2016).

The primary utility of the vapor exposure model is that dependence-like symptoms can be induced in short periods of time, relative to models that require the animal to voluntarily drink. The exposure protocol used most often involves repeated exposures to vaporized alcohol separated by withdrawal periods. Typically, this is referred to as the chronic intermittent alcohol (CIE) vapor exposure model (Griffin et al. 2009). While the exact procedure varies between laboratories, the CIE exposure procedure often consists of exposure to vaporized alcohol for a large portion of the day (12–16 h) followed by withdrawal period (8–12 h) where room air is pumped into the chamber. This procedure is repeated daily (usually 4 consecutive days) before a longer withdrawal period (often 3 days) is imposed, for a total time of 1 week (referred to as one "cycle"). The CIE procedure in mice has typically been utilized to deliver one, two, three, four, or five cycles of alcohol vapor exposure, depending on the requirements of the study (Fig. 1a). Because mice metabolize alcohol at a high rate, to achieve desired blood alcohol levels it is necessary to inhibit the metabolic pathway of alcohol, and expose the animals to a "loading" dose of alcohol. This is typically achieved via systemic injection of the

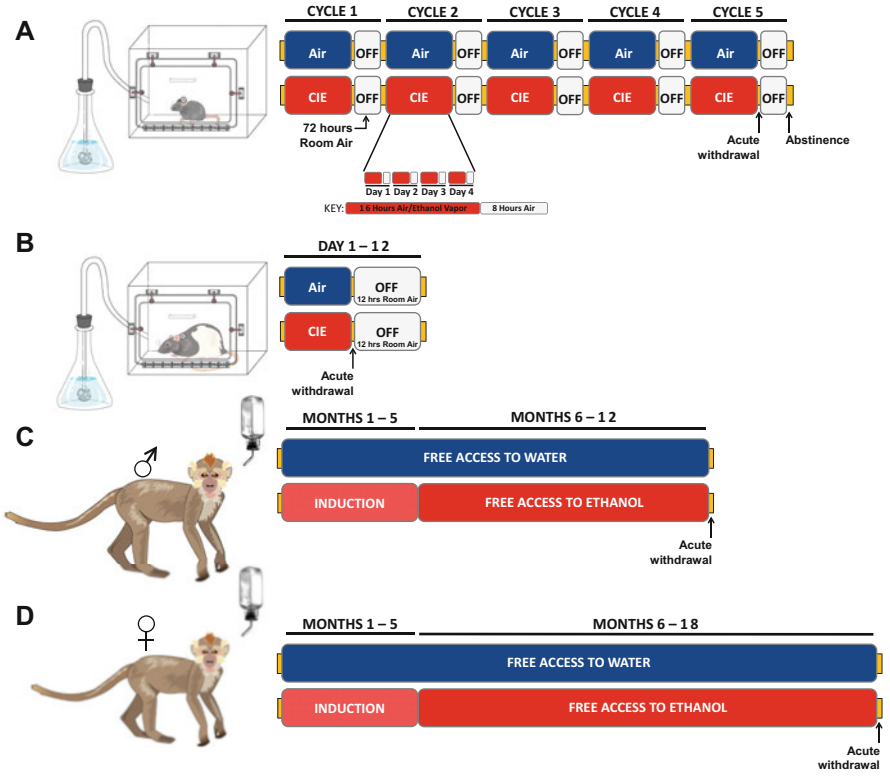

Fig. 1 Example paradigms of chronic ethanol administration across species. Schematic outline of ethanol administration in (**a**) mice, (**b**) rats, (**c**) male cynomolgus monkeys, and (**d**) female rhesus monkeys (note that many parameters vary slightly across lab and study depending on the specific hypotheses being addressed). (**a**, **b**) In order to precisely regulate blood ethanol levels and achieve high levels of ethanol intake, vaporized ethanol administration paradigms are utilized for noncontingent chronic exposure in rodents. (**a**) Mice undergo multiple cycles, ranging from 1 to 5, of intermittent ethanol vapor exposure (inset: daily ethanol exposure during one cycle) separated by 3 days of abstinence. In many of the experiments discussed in this review, behavioral and physiological measurements were taken during acute withdrawal, immediately following removal from the vapor chamber, or during abstinence – typically 72 h after last exposure. (**b**) Rats undergo a similar vapor exposure paradigm; however, daily ethanol exposure is continuous and not divided into cycles by days of abstinence. (**c**, **d**) The pattern of ethanol consumption by nonhuman primates is similar to that of humans and volitional intake paradigms offer greater insight to consummatory behaviors and subsequent physiological alterations. In many of the experiments discussed in this review, following a 5-month schedule-induced polydipsia induction period, nonhuman primates were given free access to ethanol and water for 22 h/day for either 6 or 12 months, and neurophysiological experiments examining the dopamine system are performed during acute withdrawal

alcohol dehydrogenase inhibitor, pyrazole, mixed with alcohol. In this model, it is important to include a control group that is housed in a similar chamber and given

injections of pyrazole, but not exposed to alcohol, to control for the effects of pyrazole as well as housing condition. Even though CIE exposure is a noncontingent exposure method (i.e., the animal has no choice but to be exposed to alcohol), it has been shown to drive augmented compulsive/anxiety-like behavior and an increase in alcohol drinking when animals are later given volitional access to alcohol, which suggests recapitulation of at least some aspects of alcohol dependence in humans (Anderson et al. 2016a, b; Rose et al. 2016).

Most protocols for exposing rats to alcohol vapor are very similar to mice. Rats are typically exposed to alcohol vapor for 12 h followed by 12 h of room air. This procedure is often repeated daily for 10–12 consecutive days (Gilpin et al. 2008) (Fig. 1b). Because rats do not metabolize alcohol as fast as mice, administration of pyrazole and a loading dose of alcohol is not required.

1.5.2 Chronic Alcohol Self-administration in Nonhuman Primates

Similar to rodents, there are many different procedures used to study nonhuman alcohol exposure. The most commonly used nonhuman primate model of alcohol consumption, and what we will focus on in this review, involves training animals to volitionally consume alcohol. Volitional consumption, as opposed to noncontingent exposure such as a vapor chamber or alcohol injection, is an important distinction. Indeed, humans consume alcohol volitionally, giving this approach strong face validity. Further, pattern of drug exposure and rate of onset/clearance are important factors in the pharmacological action of drugs, and can often affect the neurochemical adaptations induced by drug exposure (Allain et al. 2015; Calipari et al. 2013). Thus, allowing the animal to consume the drug in a self-imposed pattern is more likely to result in effects similar to those in humans.

The method of inducing alcohol self-administration often varies across laboratory and/or study. Generally, animals are trained to pull a lever or activate a finger-poke to receive access to a sipper containing alcohol (Grant et al. 2008; Vivian et al. 2001). In some cases, schedule-induced polydipsia is used to augment alcohol consumption during the initial exposure and training phase (Grant et al. 2008; Vivian et al. 2001). Schedule-induced polydipsia involves simply delivering small amounts of food at spaced intervals. Because most mammals tend to increase fluid consumption during times of feeding, the increase in number of feeding bouts produces a robust increase in fluid consumption (Falk 1966). Once trained to consume alcohol, animals are then allowed to drink, either freely with continuous access to alcohol in the home cage, or in daily sessions where alcohol becomes available. In this review we will primarily discuss studies in which nonhuman primates were given 22 h/day access to alcohol in the home cage, for a period of 6–18 months (Fig. 1c, d). Importantly, this model produces robust individual differences in alcohol intake between animals and between days within each animal. This allows for determining the effects of alcohol across a range of intake, as well as the factors that may predict individual differences in alcohol consumption (Cuzon Carlson et al. 2011; Grant and Bennett 2003; Grant et al. 2008; Nimitvilai et al. 2017; Pleil et al. 2015b; Vivian et al. 2001; Baker et al. 2014, 2017).

1.6 Tonic and Phasic Dopamine Signaling

Dopamine neurons originating from the VTA have two distinct types of firing patterns, tonic or phasic firing. Tonic firing is characterized by the periodic occurrence of single action potentials (2–5 Hz), while phasic firing is characterized by bursts of action potentials (10–25 Hz) occurring in close temporal proximity (Grace and Bunney 1983, 1984). These two types of signaling are critical in regulating reward processing and internal state, and can be controlled by both changes in VTA firing and local modulatory mechanisms directly at dopamine terminals in the nucleus accumbens (NAc; a subregion of the ventral striatum) (Exley and Cragg 2008). Below we will discuss the different methods for measuring tonic and phasic dopamine signaling.

Tonic dopamine levels, often referred to as extracellular levels, are comparatively low (usually 5–20 nM) and can be measured with relatively low temporal resolution over several minutes using techniques such as in vivo microdialysis. To conduct microdialysis, a concentric perforated probe is implanted into the area of interest. Artificial cerebrospinal fluid is perfused into the region of interest; neurotransmitters, such as dopamine, diffuse down their concentration gradient across this perforated membrane. This fluid is then collected over 5–30 min and analyzed using detection methods such as high performance liquid chromatography or mass spectrometry, which allow for quantification of analytes (e.g., neurotransmitters) within the sample. Changes in tonic dopamine levels have been shown to predictably alter thresholds for intracranial self-stimulation, which is used to monitor the function of brain reward systems and measure the motivational state of the animal, suggesting that tonic dopamine levels are involved in reward sensitivity and affective states (Carlezon and Chartoff 2007; Hernandez et al. 2012; Kokkinidis and McCarter 1990; Negus and Miller 2014; see Dobrossy et al. 2015 for review).

While microdialysis can give information about relative levels of synaptic neurotransmitters, it is important to understand how receptors and local regulation of dopamine neurons are influenced by alcohol exposure. Ex vivo fast-scan cyclic voltammetry (FSCV), typically performed in coronal brain slices, allows for measuring experimenter-stimulated dopamine release when the dopamine terminal is isolated from its endogenous inputs (due to severing these connections in the slicing process). FSCV detects electroactive analytes (including dopamine) by applying voltage to a microelectrode, which drives oxidation of dopamine to dopamine-o-quinone; the oxidation of dopamine results in the loss of electrons which are detected at the electrode as a change in current which is proportional to the concentration of dopamine molecules near the surface of the electrode. Thus, based on the electroactive properties of dopamine, FSCV is able to detect dopamine levels with high specificity, even within a heterogeneous environment of transmitter signaling. This detection can occur quickly (typically sampled at 10 Hz) allowing for information to be obtained about real-time dopamine release and clearance kinetics. With this technique, receptors expressed on dopamine terminals can be pharmacological targeted and their effects on the kinetics of dopamine signaling can be assessed (for review of the utility of ex vivo voltammetry see Ferris et al. 2013).

Phasic firing refers to bursts of activity that result in high concentrations (estimated to be around 100 μM) of dopamine within the synapse (Grace et al. 2007). These phasic dopamine signals are particularly important in the case of addiction where they act to encode information not just about rewards, but also about cues that predict their availability. For example, reward conditioning experiments have shown that phasic dopamine responses occur immediately following presentation of unexpected rewards; however, after multiple pairings of cue and reward, phasic dopamine responses shift to the cue predicting the reward instead of the reward itself (Phillips et al. 2003; Schultz 1998). Thus, understanding how this type of signaling is dysregulated by alcohol has implications not only for subsequent alcohol use, but also for decision-making and reward seeking outside of alcohol-related contexts.

Thus, understanding phasic and tonic dopamine signaling is crucially important as their interplay controls the execution of motivated behaviors, and the examination of these two aspects of dopaminergic signaling in tandem allows a greater understanding of the alcohol-induced maladaptive responses to external stimuli.

2 Dopamine Signaling Following Chronic Alcohol Exposure

2.1 Acute Effects of Alcohol on Dopamine Release

Acute alcohol administration has distinct, regionally specific effects on dopamine system activity. Systemic alcohol administration transiently increases extracellular tonic levels of dopamine in the NAc of rodents and monkeys as measured by microdialysis (Bradberry 2002; Karkhanis et al. 2016; Weiss et al. 1993; Yim et al. 1998). Similarly, an in vivo FSCV study in awake, behaving rats showed that alcohol administration resulted in an increase in phasic dopamine release (Shnitko and Robinson 2015). In contrast, ex vivo FSCV studies show that acute application of alcohol to brain slices results in a *reduction* of phasic dopamine release in the NAc of both rodents and monkeys (Siciliano et al. 2016b; Yorgason et al. 2014, 2015), which is dependent on alcohol concentration and frequency of stimulation. Reductions in dopamine release in these experiments were observed only at high concentrations of alcohol (80 mM and above) and during high frequency of stimulation (20 Hz and above) (Yorgason et al. 2015). While these findings may seem in opposition, the inconsistencies between in vivo (enhanced release) and ex vivo (reduced release) are likely driven by the fact that dopamine terminals are separated from the cell body in ex vivo slice preparations. In the ex vivo slice preparation, effects that are observed represent only the synaptic connections that are maintained within the slice, and do not assess the full spectrum of circuit connectivity between the region of interest and the rest of the brain. Without the contribution of alcohol-mediated excitation of VTA neurons to augment dopaminergic signaling, NAc terminals are inhibited by alcohol. However, the net effect of alcohol in the intact animal (i.e., inhibitory actions at the terminal and excitatory actions at the cell body) is increased extracellular dopamine levels in the

striatum. These findings highlight both the complexity of this system and the need for multiple levels of exploration (from the slice to the whole animal) in order to fully appreciate the pharmacological actions of alcohol on the dopaminergic system. For more information on alcohol's presynaptic actions, including non-dopaminergic systems, see Lovinger (2017) elsewhere in this volume.

2.2 Effects of Chronic Alcohol Exposure on Dopamine Release During Abstinence

While the section above outlines the acute effects of alcohol on dopamine release, chronic use leaves a lasting impact on dopamine release during alcohol-free periods, which may contribute to maladaptive decision making. In this section we will outline literature which has examined dopamine release in animals with a history of alcohol exposure, when there is no alcohol "on board" (i.e., during withdrawal).

Studies examining the role of chronic alcohol exposure on dopamine release have generally yielded mixed results, with species, sex, and experimental design appearing to have a strong influence on the findings. For example, stimulated dopamine release in ex vivo slices preparations was attenuated following three to five cycles of CIE exposure in mice (Karkhanis et al. 2015; Rose et al. 2016; but see Melchior and Jones 2017). However, in rats, shorter exposure to alcohol vapor over 5 or 10 days did not alter dopamine release as compared to control animals (Budygin et al. 2007). Data from nonhuman primates further "muddies the waters" in regard to interpreting the effects of alcohol exposure on stimulated dopamine release. In contrast to the decreased release observed in mice, male cynomolgus macaques were found to have increased stimulated dopamine release following 6 months of alcohol self-administration (Siciliano et al. 2015a). Complicating matters further, female rhesus macaques showed no change in dopamine release after 12 months of alcohol self-administration (Siciliano et al. 2016b). The driving factor underlying the inconsistency within the nonhuman primate studies is currently unclear, as sex, length of exposure (6 vs 12 months), and species (cynomolgus vs rhesus) were all divergent between the two studies.

Although these seemlying inconsistent results may suggest that alterations in dopamine release are not related to the primary pathology of AUD, it should also be noted again that ex vivo measurements of dopamine release can only give insight into certain aspects of the system. Indeed, many in vivo studies of dopamine release in response to stimuli have demonstrated that release can encode many different aspects of learned behaviors and drug associated cues, and is plastic throughtout the formation of these associations (Wanat et al. 2009). Because the afferent inputs that drive dopamine release in vivo are severed in an ex vivo slice preparation, ex vivo approaches provide insight to the size of the readily reasable pool of dopamine, but do not capture the complexity of dopaminergic encoding of these behaviors. It is also important to note that the tonic, extracelluar level of dopamine is depedent on many factors (discussed below); when tonic dopamine levels are measured via microdialysis, multiple studies have found them to be decreased following alcohol

exposure in rats (Rossetti et al. 1992, 1999). Further, metabolic markers of dopaminergic activity are reduced in macaques after chronic alcohol use (Cervera-Juanes et al. 2016).

2.3 Dopamine Uptake

Extracellular dopamine levels are a complex interaction between dopamine release and its reuptake via the dopamine transporter (DAT) (Ferris et al. 2014). Not only is the DAT a major factor in determining tonic extracellular levels, but it is also thought to tightly regulate the "sphere of influence" and duration of dopamine effects at postsynaptic receptors as it flows out of release sites (Cragg and Rice 2004). Thus, DAT function and expression is an integral component in guiding dynamic dopamine neurotransmission.

Exposure to chronic alcohol modulates the DAT, both in terms of its function and expression. In mice, repeated cycles of CIE exposure augments dopamine reuptake, which is a primarily DAT mediated process, in the NAc (Karkhanis et al. 2015, 2016; Rose et al. 2016; Melchior and Jones 2017). In CIE models, uptake rate is enhanced immediately following cessation of the final alcohol exposure and are maintained for at least until 72 h into withdrawal, suggesting that this effect may be long lasting, though later time-points have not yet been tested (Karkhanis et al. 2015; Rose et al. 2016) (Fig. 2). Further, DAT density in the NAc is increased after CIE (Healey et al. 2008). Enhanced dopamine uptake rate likely contributes to reduced tonic dopamine levels by increasing the speed of dopamine removal from the extracellular space.

Enhanced dopamine uptake rate following alcohol exposure is a phenomenon that is strongly conserved across species. Similar to mice, dopamine uptake rate is increased in rats exposed to CIE vapor (Budygin et al. 2007). In rats exposed to an alcohol-containing liquid diet for 1 year, DAT protein expression was increased in both the ventral and dorsal striatum (Rothblat et al. 2001), suggesting that increased functional uptake is a result of increased protein levels, although other mechanisms such as conformational alterations or changes in the affinity state of the DAT could also be at play. These findings have been extended to nonhuman primates, where dopamine uptake rate has been found to be increased in the NAc of male cynomolgus macaques and female rhesus macaques after 6 and 12 months of volitional access to alcohol, respectively (Siciliano et al. 2015a, 2016b). Together, these data demonstrate that, in the NAc, dopamine uptake is increased across species and experimental setting, suggesting that it may be an important factor in driving the core symptomology of AUD.

One exception is that in male cynomolgus macaques, uptake rate in the dorsolateral caudate (a subregion of the striatum typically thought to be involved in motor control and habit learning (Graybiel 1995, 2008; Porrino et al. 2004) were *reduced* after chronic access to alcohol (Siciliano et al. 2015a)). This decrease in dopamine uptake rate likely produces increases in extracellular dopamine levels. Interestingly, the ratio of uptake rates between the dorsolateral caudate and NAc was highly

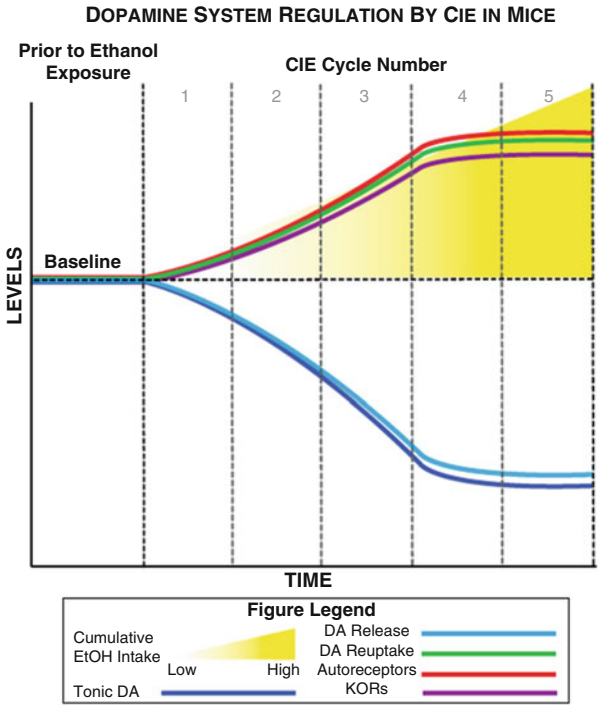

Fig. 2 Alterations in NAc dopamine signaling in mice following successive cycles of CIE. This timeline represents schematized alterations in dopamine system regulation with increasing exposures to chronic intermittent ethanol cycles. With increasing cumulative ethanol exposure, tonic dopamine levels (measured via microdialysis) and stimulated dopamine release (measured via FSCV) are diminished, but dopamine uptake rate is enhanced, resulting in faster clearance of dopamine from the synaptic cleft. In addition, KOR and D2/D3 autoreceptor function is also augmented. Together these factors give rise to a systemic hypodopaminergic state in animals exposed to chronic intermittent ethanol. These effects appear to be dose-dependent on total cumulative ethanol exposure until they plateau after approximately three to five cycles. *DA* dopamine, *EtOH* ethanol, *KORs* kappa opioid receptors

correlated with alcohol intake across animals. While the behavioral relevance of differential changes in dopamine uptake between these two regions remains to be determined, there is evidence that dysregulation of dopamine-mediated communication between these areas can lead to habitual and addiction-like behaviors (Belin and Everitt 2008; Everitt and Robbins 2013). Thus, enhancement of NAc dopamine reuptake and blunting of dorsolateral caudate dopamine reuptake together may contribute to maladaptive alcohol seeking and intake following chronic exposure.

Not only is dopamine uptake altered by chronic alcohol administration in preclinical models, there is also evidence that abnormal dopamine uptake may contribute to disease states in the human population. Genetic association studies point to alterations in the DAT in a subset of individuals with an AUD diagnosis, which may confer a heightened vulnerability towards development of the disease (Köhnke et al.

2005). The relationship between DATs and alcoholism in humans is not entirely clear, however. Alcoholism is associated with both increases and decreases in DAT availability as measured by positron emission tomography (PET) or single photon emission computed tomography (SPECT), depending on the study (Laine et al. 1999; Tiihonen et al. 1995; Tupala et al. 2006; Yen et al. 2015, 2016). These discrepancies may be due, at least in part, to different durations of withdrawal between studies and limitations of lower resolution, noninvasive approaches which are typically employed in human studies. However, given the abundant preclinical data showing alterations in uptake rate following chronic alcohol exposure discussed above, and implications for genetic DAT alterations in the clinical population, it is clear that the DAT plays a significant role in the etiology of AUD that should be further examined in future studies.

2.4 Autoreceptors

In the NAc, dopamine D2-type (D2, D3, D4) receptors are found on medium spiny projection neurons, local interneurons, and presynaptic terminals from afferent inputs (Alcantara et al. 2003; Ford 2014; Levey et al. 1993). D2-type receptors that are located on presynaptic dopamine terminals are autoreceptors, which function in a feedback-inhibitory manner, binding released dopamine and inhibiting future release. D2-type dopamine autoreceptors are G-protein coupled receptors expressed at both cell bodies and presynaptic terminals of dopamine neurons, where they inhibit action potential firing activity, release and synthesis of dopamine. Thus, in the NAc, when extracellular dopamine levels are high, autoreceptors inhibit dopamine release and synthesis, driving the system towards homeostasis. For this reason, D2-type autoreceptors are often conceptualized as the "brakes" on the dopamine system. Of the members of the D2-type receptor family, D2 receptors themselves have been found to mediate the majority of autoreceptor activity in the striatum, although D3 and D4 receptors are also present (Bello et al. 2011; Meador-Woodruff et al. 1994; Meller et al. 1993; Rubinstein et al. 1997).

Most studies examining D2-type autoreceptor sensitivity following chronic alcohol exposure have shown functional increases in activity/sensitivity, contributing to a reduction in dopamine signaling. Multiple cycles of CIE exposure result in greater autoregulation of release in the NAc in mice (Karkhanis et al. 2015), but shorter exposure times did not change the ability of autoreceptors to inhibit dopamine release in rats (Budygin et al. 2007) or their ability to inhibit dopamine synthesis in mice (Siciliano et al. 2017). Repeated CIE-induced increases in autoreceptor sensitivity appears to be relatively short-lived during abstinence, however, with sensitivity returning to control levels within a few days (Karkhanis et al. 2015). Typically, the sensitivity of these receptors has been assessed by examining the ability of D2-type dopamine receptor agonists to inhibit dopamine release ex vivo. When greater effects of D2-type specific agonists are observed, it is interpreted as an increase in the sensitivity of these receptors, which translates to increased inhibitory feedback when endogenous dopamine interacts with these receptors. Thus, increased

sensitivity of D2-type autoreceptors likely contributes to a hypodopaminergic state via decreased probability of dopamine release from presynaptic terminals.

Similar findings have been observed in nonhuman primate models of AUD. In one study of male cynomolgus macaques it was found that, after 6 months of volitional alcohol drinking, there was no change in overall autoreceptor-mediated inhibition of dopamine release; however, there was a shift in the relative contribution of D2 vs D3 dopamine autoreceptors towards D2 receptors (Siciliano et al. 2016b). In other words, D2 sensitivity was increased, and D3 sensitivity was decreased such that the sum of autoreceptor inhibition remained the same while the contribution of the two receptor subtypes was shifted. In two studies of monkeys exposed to longer, 12- to 18-month periods of alcohol drinking, D2-type autoreceptor sensitivity was increased, but the relative contributions of receptor subtypes were not queried (Budygin et al. 2003; Siciliano et al. 2016a). Thus, it appears that, across species, overall changes in autoreceptor sensitivity occur after extended exposure to alcohol, but not after modest exposure lengths. These consistent cross-species findings of dopamine autoreceptor changes, with consistent relative time-courses and direction of change towards greater dopamine inhibition, provide confidence that autoreceptor changes may be functionally related to the core symptomology of AUD.

2.5 Kappa Opioid Receptors

2.5.1 Dopamine and Kappa Opioid Receptor Interactions

Like D2/D3 autoreceptors, kappa opioid receptors (KORs) are located on dopamine terminals (Ebner et al. 2010; Svingos et al. 2001; Werling et al. 1988) and act to reduce dopamine release. KOR activation results in reduced tonic dopamine levels as well as both decreased probability and magnitude of phasic release events (Steiner and Gerfen 1996). Given the role of dopamine in reward, it is not surprising that the activation of KORs, which inhibit dopamine release, is generally aversive (Land et al. 2009). Dynorphin, the endogenous ligand of KORs, is released during exposure to painful, noxious, or stressful stimuli (Bruchas and Chavkin 2010; Land et al. 2009; Nabeshima et al. 1992). KORs are believed to exert their behavioral and subjective effects in part through inhibition of dopaminergic signaling (Ebner et al. 2010; Svingos et al. 2001; Werling et al. 1988).

In the NAc, dopamine, dynorphin, and KORs have an intimate relationship of interconnectivity and feedback regulation. GABAergic medium spiny neurons (MSNs) account for more than 90% of all neurons in the striatum and are the major projection population. Striatal MSNs consist of two distinct populations, which are interspersed and equally distributed across the dorsal and ventral striatum, and differentiated based on the expression of dopamine receptors and opioid peptides. Approximately half of the MSN population expresses excitatory D1-type dopamine receptors and dynorphin peptides, and the other half of MSNs express inhibitory D2-type receptors and enkephalin peptides (ligand for mu and delta opioid receptors) (Gerfen et al. 1990). Dynorphin is generated in D1-MSNs in response to D1 receptor activation, and its release inhibits further dopamine release from the

presynaptic terminal (Gerfen and Surmeier 2011; Steiner and Gerfen 1996). Dynorphin peptides are transported to recurrent collateral axons within the NAc and decrease dopamine release via presynaptic KORs (Steiner and Gerfen 1993). In this way, a feedback loop wherein dopamine release can increase the probability of dynorphin release, which in turn reduces dopamine release, is propagated.

2.5.2 Kappa Opioid Receptors and Chronic Alcohol Exposure

In humans, activation of KORs has been associated with feelings of dysphoria, and both KORs and dynorphin mRNA are upregulated in patients suffering from AUD (Bazov et al. 2013). In preclinical models, a single injection of alcohol in naïve rats results in a transient increase in dynorphin levels (Kuzmin et al. 2013; Lam et al. 2008; Marinelli et al. 2006). Following CIE exposure, inhibition of KORs has been shown to successfully mitigate negative affect-like behavioral alterations observed in mice and rats, without altering these behaviors in alcohol-naïve animals (Anderson et al. 2016b; Pleil et al. 2015a; Rose et al. 2016; Kissler et al. 2014). Interestingly, the effect of KOR activation on alcohol intake behaviors appears to be strongly influenced by the animal's history of alcohol intake and state of dependency. Pharmacological blockade of KORs does not change alcohol consumption in non-alcohol-dependent animals; however, in animals that have been made dependent through CIE exposure, and show high dependence-induced volitional alcohol intake, KOR blockade reduces alcohol consumption to control levels (Rose et al. 2016; Walker et al. 2011; see Anderson and Becker 2017 for review). Conversely, in naïve animals, pretreatment with KOR agonists drives an increase in alcohol consumption comparable to animals that have been previously made dependent, again suggesting that alcohol-induced increased activity of these receptors is associated with excessive alcohol intake (Anderson et al. 2016b; Rose et al. 2016).

Behavioral studies, outlined above, have suggested that KORs are functionally altered by previous alcohol exposure, and subsequent studies directly measuring KOR regulation of dopamine signaling after chronic alcohol exposure support this hypothesis (Karkhanis et al. 2016; Rose et al. 2016; Siciliano et al. 2016a). Indeed, in mice and rats, the dopamine-decreasing effects of KOR activation, observed using ex vivo FSCV, are heightened dramatically following CIE exposure (Karkhanis et al. 2016; Rose et al. 2016). Thus, alcohol exposure augments the ability of KORs to reduce dopamine, contributing further to hypodopaminergia following chronic alcohol exposure. Importantly, dependence-induced increases in alcohol consumption can be reduced via microinfusion of a KOR antagonist directly into the NAc (Nealey et al. 2011). This suggests a causal role for increased KOR function in the NAc in aberrant alcohol consumption.

Augmentation of KOR sensitivity is conserved across species. Following 6 months of drinking in male cynomolgus macaques, KOR-mediated inhibitory regulation of dopamine signaling in the NAc is increased (Siciliano et al. 2016a). Further, KOR-mediated inhibition of dopamine release in the dorsolateral caudate is augmented after alcohol drinking (Siciliano et al. 2015a). Interestingly, while both regions appear to develop KOR hyper-function, KOR activity in the NAc, but not the dorsolateral caudate, is correlated with alcohol intake (Siciliano et al. 2015a), again

suggesting that KOR signaling in the NAc is a critical node in driving excessive alcohol intake. Further, these effects are consistent across sex, as 1 year of alcohol self-administration also increased KOR regulation of dopamine release in the NAc of female rhesus macaques (Siciliano et al. 2016a). Together, these studies demonstrate that KOR regulation of dopamine release is increased across species as well as several other experimental parameters, including route of administration, exposure length, and withdrawal time, suggesting an integral role in the etiology of AUD. We hypothesize that alcohol dependence-induced increases in KOR function contribute to a hypodopaminergic state possibly leading to craving and excessive consumption of alcohol. These alterations in KOR changes at the dopamine terminal are illustrated in Fig. 3.

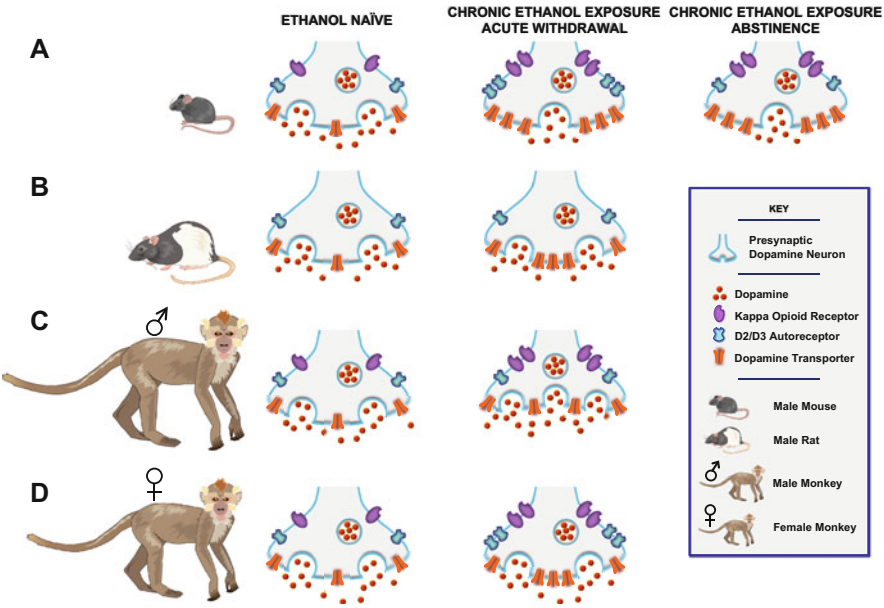

Fig. 3 Putative synaptic alterations associated with chronic ethanol exposure across species. Synaptic changes in the NAc associated with withdrawal from chronic alcohol exposure in (**a**) male mice, (**b**) male rats, (**c**) male cynomolgus monkeys, and (**d**) female rhesus monkeys. (**a**) In mice, acute withdrawal from chronic intermittent ethanol exposure is associated with reduced dopamine release and augmented reuptake, D2R/D3R autoreceptor, and KOR sensitivity. With the exception of autoreceptor sensitivity, most of these effects are relatively long lasting, and remain altered 72 h following the final ethanol exposure. (**b**) In rats, dopamine release and autoreceptor function are unaffected during acute withdrawal; however, enhanced D1R and DAT sensitivity are observed. (**c**) Male cynomolgus monkeys show synaptic alterations remarkably similar to male mice during acute withdrawal from chronic ethanol intake, whereby DAT and KOR functions are augmented. However, dopamine release is increased and total autoreceptor function remains unaltered. (**d**) In female rhesus monkeys, while there is no change in dopamine release, the DAT, KOR, and autoreceptor function in increased during acute withdrawal

The extensive alcohol-induced reductions in presynaptic dopamine terminal activity in the NAc would be predicted to result in differential signaling onto downstream, postsynaptic targets. In the NAc, these consist primarily of D1 and D2 receptor expressing MSNs, which are also altered in AUD models. These other aspects of alcohol-induced alterations in striatal activity are beyond the scope of the current chapter, but have been extensively characterized elsewhere and continue to be areas of intense investigation (Clarke and Adermark 2015; Engel and Jerlhag 2014; Koob 2014; Renteria et al. 2017; Soderpalm et al. 2009; Tupala and Tiihonen 2004).

3 Conclusions

It is clear that factors such as alcohol exposure length, species, route of administration, and withdrawal time-point can be important variables in determining the effects of alcohol on the dopamine system. However, in this chapter, we highlight some of the robust findings that transcend these variables and may hint at common neurobiological substrates following chronic exposure to alcohol. Primary examples of these commonalties include increased functionality/sensitivity of multiple negative regulators of dopamine signaling, such as DATs, dopamine autoreceptors, and KORs. The combination of increased clearance of dopamine from the synapse, via upregulation of DATs, with enhanced KOR and autoreceptor function, likely combines to produce hyper-inhibitory regulation of dopamine signaling. Thus, deviations from "healthy" function of the mesolimbic dopaminergic system following chronic exposure to alcohol appear to be a universal adaptation that may be driving maladaptive behaviors associated with repeated alcohol exposure.

The use of multiple species and paradigms offers insight into possible treatment strategies that could alleviate suffering in individuals with an AUD. As illustrated above, such diverse experimental designs help to "separate the wheat from the chaff" by identifying consistent, key neurobiological changes following exposure. To this end, we argue that successful pharmacological strategies may lie in a combinatorial pharmacotherapy that would quiet both DAT and KOR systems (e.g., a DAT inhibitor and KOR antagonist or partial agonist). Such a treatment could suppress two key contributors to dopamine signal reduction following chronic alcohol exposure, and may alleviate negative affective states that are pervasive in abstinent AUD patients. Resultant normalization of affect during withdrawal would be predicted to both improve quality of life and decrease likelihood of relapse in individuals striving to maintain abstinence.

Taken together, it is clear that key alterations in mesolimbic dopamine signaling are consistently observed following chronic alcohol exposure across species and administration paradigms. Although there is far more work to be done in order to fully elucidate the role of dopaminergic signaling in neural and behavioral adaptations following alcohol exposure, pharmacological strategies targeting these adaptations are a potentially important treatment avenue for alleviating suffering in individuals with an AUD.

Acknowledgments This work was funded by NIH grants U01 AA014091, R01 AA021099, P01 AA023299 (SRJ), T32 AA007565 (CAS, ANK, KMH, JRM), F31 DA037710, F32 MH111216, Brain and Behavior Research Foundation (CAS), K01 AA023874 (ANK), and F31 AA023144 (JRM).

Financial Disclosure: The authors declare no competing financial interests.

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Central Noradrenergic Interactions with Alcohol and Regulation of Alcohol-Related Behaviors

Elena M. Vazey, Carolina R. den Hartog, and David E. Moorman

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Abstract

Alcohol use disorder (AUD) results from disruption of a number of neural systems underlying motivation, emotion, and cognition. Patients with AUD exhibit not only elevated motivation for alcohol but heightened stress and anxiety, and disruptions in cognitive domains such as decision-making. One system at the intersection of these functions is the central norepinephrine (NE) system. This catecholaminergic neuromodulator, produced by several brainstem nuclei, plays profound roles in a wide range of behaviors and functions, including arousal, attention, and other aspects of cognition, motivation, emotional regulation, and control over basic physiological processes. It has been known for some time that NE has an impact on alcohol seeking and use, but the mechanisms of its influence

E. M. Vazey (✉) · C. R. den Hartog
Department of Biology & Neuroscience and Behavior Graduate Program, University of Massachusetts Amherst, Amherst, MA, USA
e-mail: evazey@umass.edu

D. E. Moorman
Department of Psychological and Brain Sciences & Neuroscience and Behavior Graduate Program, University of Massachusetts Amherst, Amherst, MA, USA

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_108

are still being revealed. This chapter will discuss the influence of NE neuron activation and NE release at alcohol-relevant targets on behaviors and disruptions underlying alcohol motivation and AUD. Potential NE-based pharmacotherapies for AUD treatment will also be discussed. Given the basic properties of NE function, the strong relationship between NE and alcohol use, and the effectiveness of current NE-related treatments, the studies presented here indicate an encouraging direction for the development of precise and efficacious future therapies for AUD.

Keywords
Adrenergic receptors · Allostasis · Dependence · Locus coeruleus · Nucleus tractus solitarius · Reward · Withdrawal

1 Introduction

Norepinephrine (NE) is a key neuromodulator in the CNS that originates from several hindbrain nuclei and projects widely across the brain. NE alters neural responsiveness in its targets, modifying activity of ongoing neural processes through pre- and postsynaptic G-protein-coupled receptors. As such, it can exert wide-reaching and complex influences over neural circuits involved in the regulation of alcohol-related behaviors. In the following chapter we discuss central noradrenergic systems, with a particular focus on NE from the locus coeruleus (LC) and nucleus tractus solitarius (NTS), their interactions with alcohol, and NE as a therapeutic target for alcohol use disorder (AUD).

2 Central Norepinephrine Systems

Central noradrenergic neurons in several small nuclei of the medulla and pons (A1–A7) use the enzyme dopamine-β-hydroxylase (DBH) to convert dopamine (DA) to NE for release (Dahlstrom and Fuxe 1964). Although their cell bodies number only in the thousands these neurons reach almost every region of the CNS through an extensively branching efferent network (Fuxe 1965). The largest population of noradrenergic neurons is the bilateral LC (A6) and subcoeruleus (A4) complex in the dorsal pons (Swanson and Hartman 1975). This population contains around half of all central NE neurons with each LC comprising ~1,500 neurons per side in rodents (Swanson 1976) and up to 50,000 per side in humans (Baker et al. 1989). Other noradrenergic nuclei are found within the lateral tegmental system that extends from the caudal midbrain and pons (A5, A7) through the medulla (A1–A3) (Moore and Bloom 1979).

In addition to descending spinal cord projections and local brainstem projections critical for autonomic regulation, NE populations show a diverse topography. The LC provides the vast majority of NE input to cerebral cortex, hippocampus,

thalamus, and cerebellum, as well as innervating basal forebrain limbic structures including the amygdala, dorsal BNST, and some parts of the hypothalamus (Moore and Bloom 1979). Non-LC NE neurons, particularly those located in A1/A2 clusters in the medulla including the NTS (A2), provide input to homeostatic and limbic regions of the forebrain including the hypothalamus, amygdala, and extended amygdala, particularly the BNST (Moore and Bloom 1979). Although contributions of other NE nuclei cannot be ruled out, the majority of studies to date investigating the relationship between NE and alcohol use have focused on signaling emanating from either the NTS or the LC.

NTS and LC receive substantial convergent input, integrating information sent from multiple forebrain, thalamic and hypothalamic, midbrain, and brainstem nuclei, in addition to receiving inputs from spinal cord and cranial nerves (Cedarbaum and Aghajanian 1978; Rinaman 2011; Takigawa and Mogenson 1977). LC inputs include also ascending NE from the NTS (Levitt and Moore 1979). These afferents from diverse regions regulate NE neurons using a range of transmitters and modulators including glutamate and GABA, NE itself, and neuropeptides such as dynorphin (DYN), corticotropin releasing factor (CRF), pituitary adenylate cyclase-activating peptide (PACAP), and orexin/hypocretin, making NE neurons directly sensitive to multiple levels of modulation (Ennis and Aston-Jones 1988; Olpe and Steinmann 1991; Valentino and Foote 1988). Of particular relevance to alcohol-associated studies, the NTS and LC are densely interconnected with frontal and insular cortex, amygdala, extended amygdala, and hypothalamus (reviewed in Berridge and Waterhouse 2003; Rinaman 2011). Finally, NE neurons co-express several additional neurotransmitters, modulators, and peptides including glutamate, neuropeptide Y (NPY), and galanin among a number of other peptides (Melander et al. 1986; Rinaman 2011; Sawchenko et al. 1985; Stornetta et al. 2002). Thus, the medullary and brainstem NE populations are strongly regulated by a diverse set of inputs and, in return, project broadly, signaling with a complex conjunction of neurochemicals.

As noted above, NE neurons project widely across the extent of the brain, targeting cortical and subcortical regions. NE projections reach the forebrain primarily through two major ascending tracts, specifically the dorsal noradrenergic bundle (DNAB) from the LC and the ventral noradrenergic bundle (VNAB) from medullary NE neurons, including NTS. These tracts then converge within the medial forebrain bundle before reaching the hypothalamus and extended amygdala (Moore and Bloom 1979). An individual NE neuron may produce up to 30 cm of terminal arbors with ~100,000 axonal varicosities (Moore and Bloom 1979). NE is released extrasynaptically from these varicosities, in addition to synaptic release at axon terminals, further expanding the influence of NE through volume transmission (Aoki et al. 1998).

The distribution of norepinephrine projections allows these neurons to play a wide and diverse role in modulating key processes throughout the brain. The vast majority of NE efferents to cognitive and sensory cortical regions comes from LC, from which NE can modulate perception and decision making in response to alcohol-related stimuli (Aston-Jones and Cohen 2005; Berridge and Waterhouse

2003). LC also provides noradrenergic, and dopaminergic (Takeuchi et al. 2016), regulation to the hippocampus where it regulates memory function, including consolidation (Sara 2015). LC and medullary NE nuclei including NTS (and A1) project to paraventricular nucleus (PVN) of the hypothalamus where they regulate HPA signaling critical to ethanol responses (Moore and Bloom 1979). In each of these regions NE modulation provides a potent influence over physiological and behavioral responses to alcohol.

NE innervation of the forebrain is also well positioned to regulate motivational and emotional responses that drive alcohol consumption and craving. NE has a potent regulatory influence over limbic circuits, including control of anxiety and stress responses through LC inputs to basolateral amygdala, inputs to BNST from NTS, and to a lesser degree LC, in addition to reciprocal connections of both regions with central amygdala (Daniel and Rainnie 2016; Park et al. 2009; Phelix et al. 1992; Valentino and Van Bockstaele 2008). NE may also impact motivational drive for alcohol through modulation of lateral hypothalamus, ventral tegmental area, and other regions of the extended amygdala which are innervated by both LC and NTS NE (Moore and Bloom 1979). NTS NE additionally provides noradrenergic tone to the nucleus accumbens, an area heavily implicated in motivation for natural and drug rewards, including alcohol (Berridge et al. 1997; Chang 1989; Wang et al. 1992).

2.1 Central Norepinephrine Functions

The canonical role of NE modulation in these target regions is to enhance neuronal responses to other synaptic inputs. The enhancement can take the form of an inhibitory suppression of baseline noise, with or without a potentiation of discrete excitatory responses. NE achieves this complex modulatory regulation through several postsynaptic receptors; excitatory Gq-mediated α1 receptors, Gs-mediated β receptors (both β1 and β2 are prominent in CNS), or inhibitory Gi α2 receptors (MacDonald et al. 1997; Morrow and Creese 1986; Nicholas et al. 1993a, b). α2 receptors also play a prominent role in regulating NE transmission presynaptically by acting as autoreceptors on NE terminals and via local collaterals in NE nuclei (Aghajanian et al. 1977; Aoki et al. 1994). NE-mediated baseline suppression and excitatory potentiation can both lead to enhanced signal-to-noise of acute signaling at NE targets. The postsynaptic mechanisms of NE vary by target region with NE acting on target cells directly, or indirectly via postsynaptic GABAergic interneurons (Bevan et al. 1973; Foote et al. 1975; Waterhouse et al. 1980). In some target regions, such as the BNST, the presence of Gi-coupled α2 receptors on postsynaptic target cells or non-NE axon terminals predominates a net inhibitory effect upon NE release, suppressing both basal activity and incoming glutamatergic signaling (Daniel and Rainnie 2016; Shields et al. 2009).

The LC-NE system exerts profound influence on arousal, cognition, and behavioral regulation and is well poised to regulate responses to alcohol-related stimuli. A key feature of LC-NE signaling is dynamic modulation both through tonic baseline neuronal activity changes and through phasic bursting activity and NE release in

response to behaviorally relevant or salient stimuli (Aston-Jones and Cohen 2005). Tonic LC neuron firing rates vary with arousal and are strongly influenced by peptidergic inputs, including stress-associated corticotrophin releasing factor (CRF) and dynorphin (DYN) from regions such as hypothalamus and extended amygdala (Valentino and Van Bockstaele 2008). In contrast, phasic responses are typically associated with salient stimuli, including conditioned salience, predictive cues, and aversive stimuli that require behavioral responses (Aston-Jones and Bloom 1981; Clayton et al. 2004; Kalwani et al. 2014).

The LC plays a major role in cognitive control. Physiological, pharmacological, and neurochemical techniques show that NE is associated with memory consolidation and executive function including response inhibition and behavioral flexibility, among others (reviewed by Aston-Jones and Cohen 2005; Robbins and Arnsten 2009). In addition to regulating cognitive function via PFC (and other areas), LC-NE plays a critical role in stress responses. LC neurons are reactive to both acute and chronic stress, in part due to strong CRF input (Valentino and Van Bockstaele 2008). CRF serves as a feedback mechanism whereby stress-related signaling dynamically regulates LC activity and, consequently, NE input back to stress-reactive systems (Van Bockstaele et al. 2001). Acute low level stress transiently increases tonic LC activity to facilitate alertness and scanning attention (Aston-Jones and Cohen 2005; Valentino and Van Bockstaele 2008). Exposure to prolonged, chronic stress dysregulates LC-NE function, and CRF mediated LC tone in a sex- and stressor-specific manner (Bangasser et al. 2010). We do not yet know if sex-dependent regulation of LC-NE function impacts stress and alcohol interactions, although recent evidence suggests this is likely (Retson et al. 2015).

Within stress circuits, the LC sends robust projections to the basolateral amygdala (BLA) (Asan 1998; Jones and Moore 1977). BLA, LC, and other inputs regulate CRF containing neurons in the central amygdala (CeA). The CRF afferents from CeA innervate many regions, including LC, potentiating stress responses (Cui et al. 2015; Gilpin 2012). Stress-mediated dysregulation of LC, in addition to producing a feed-forward enhancement of anxiety and stress, disrupts NE regulation of cognitive function in PFC.

Medullary NE has a profound influence on homeostatic functions and emotional regulation and plays a prominent role in motivational drive related to the seeking of alcohol and other drugs of abuse (Rinaman 2011; Smith and Aston-Jones 2008). The A2 noradrenergic population plays a role in both the execution and inhibition of feeding behaviors, though the preponderance of studies to date emphasizes its role in suppression of feeding (Rinaman 2010, 2011; Roman et al. 2016; Wellman 2000). The NTS also plays an important role in emotional regulation and motivated behavior, in part through its connections with the NAc, CeA, PVN, and BNST (Delfs et al. 1998; Rinaman 2011; Sawchenko and Swanson 1981, 1982; Smith and Aston-Jones 2008). The main outcome of NTS innervation of such areas is primarily increased stress and behavioral inhibition, and some investigators have considered the NTS projections to limbic targets to be main activators of aversive emotional state, in contrast with LC-NE, the activation which ultimately promotes arousal and exploratory behavior (Rinaman 2011). In all likelihood both systems contribute to

stress and anxiety, but the potent innervation of limbic structures such as those noted above, and the plethora of studies demonstrating a particularly influential role of the NTS over the HPA axis and behavioral components of stress indicate particularly privileged role for this system (Herman 2017; Rinaman 2011).

In general NE is broadly involved in multiple aspects of motivation, including driving behaviors associated with drugs of abuse. The history and current understanding of the role of NE in drug seeking have been the subject of a number of excellent recent reviews (Espana et al. 2016; Fitzgerald 2013; Smith and Aston-Jones 2008; Sofuoglu and Sewell 2009; Weinshenker and Schroeder 2007; Zaniewska et al. 2015). Although early studies of the neural circuitry of drug abuse highlighted a prominent role for NE, research gradually shifted focus to DA as a final common pathway underlying addiction (Weinshenker and Schroeder 2007). However, recent studies have demonstrated the importance of NE across multiple classes of drugs of abuse (psychostimulants, opiates, etc.), particularly with respect to relapse behaviors. The details underlying such lines of research are too extensive and diverse to consider here and, in focusing exclusively on the role of NE in alcohol-related behaviors and AUD, we refer readers to the reviews above for more detailed consideration.

3 Norepinephrine and Alcohol

The NE system in general is highly responsive to ethanol, and there is a growing appreciation that noradrenergic signaling may underlie substantial components of both controlled and excessive drinking. There have even been proposals that NE is more critical than dopamine (DA) for ethanol reward (Amit and Brown 1982). Due in part to the organization and functions of noradrenergic systems described above, as well as effects of NE manipulation on alcohol seeking, described below, NE signaling has long been posited as a key neural mechanism involved in both positive and negative motivation for alcohol use (Koob 2014).

3.1 Changes in Noradrenergic Function Mediated by Acute and Chronic Alcohol

In early human studies, acute ethanol produced increases in NE and the NE metabolite 3-methoxy-4-hydroxy-phenylglycol (MHPG) measured in CSF (Borg et al. 1981) and plasma (Howes and Reid 1985). The acute increases in central NE were greater in alcoholic patients than in healthy controls and were correlated with blood alcohol levels. The increased levels observed in patients decreased significantly after multiple days of abstinence. These findings suggested that acute ethanol elevates the activity of central NE neurons, and the LC was proposed as a site of action (Borg et al. 1981). Further studies comparing MHPG levels between alcohol-dependent patients and healthy controls were inconclusive (Petrakis et al. 1999) and potentially

depend on withdrawal state, indicating that further direct studies of central NE function are warranted.

Early investigations of NE and alcohol in animal models focused on the impact of alcohol administration on catecholamine metabolism. These studies found consistent changes in NE function during acute intoxication. At doses ranging from 1 to 5 g/kg of ethanol, brain NE content was reduced alongside increases in NE metabolites, specifically 3,4-dihydroxyphenylglycol (DHPG), and vanillomandelic acid (VMA). This was seen in both outbred and alcohol preferring P rats (Alari et al. 1987a; Karoum et al. 1976; Murphy et al. 1983). These increases in NE turnover during acute intoxication normalized 6 h after ethanol exposure and were consistently more pronounced than changes in dopamine turnover, particularly at lower doses of ethanol (Corrodi et al. 1966). Whether the increased turnover results from increased vesicular leakage or synaptic NE release remains unclear given conflicting findings from various approaches used to measure neuronal activity.

The idea that increased NE release and turnover is due to enhanced synaptic release is supported by increases in neuronal activation, often measured with c-Fos expression, within NE populations after acute ethanol exposure. c-Fos is an immediate early gene marker upregulated by neuronal activity and used as a post hoc proxy of recent activation (Dragunow and Faull 1989). Several studies have found an upregulation of c-Fos activity specifically in tyrosine hydroxylase (TH) or DBH positive neurons within the LC, RVLM (A1/C1) and NTS after intragastric or intraperitoneal injection of ethanol (Lee et al. 2011; Thiele et al. 2000). After high doses of acute ethanol, elevations in LC c-Fos are more pronounced in alcohol non-preferring strains than alcohol preferring strains of rats (Thiele et al. 1997). In the NTS, ethanol enhancement of GABAergic transmission, indirectly resulting in disinhibition of local TH positive neurons including NE, has been proposed as a mechanism for c-Fos induction by high doses in vivo (4 g/kg) (Aimino et al. 2017). A similar mechanism of disinhibition in LC-NE neurons is plausible, although GABAergic interneurons are not widely interdigitated within the nucleus. A pool of GABA neurons within the dendritic fields of LC, however, would be capable of providing potent local regulation (Aston-Jones et al. 2004).

Direct recordings of NE neurons after acute alcohol have been made to confirm changes in activity indicated by NE turnover and c-Fos. The complexity of noradrenergic interactions with ethanol is highlighted by differential findings between electrophysiological studies and other measures neuronal activation. The majority of electrophysiological studies measuring responses of NE neurons to acutely administered ethanol have targeted LC-NE neurons, though similar effects have been demonstrated in an unidentified neuron population in NTS (Aimino et al. 2017). Systemic and direct local administration of relatively high doses of ethanol has elicited either no change, or suppression in LC unit activity via an enhancement of inwardly rectifying potassium currents (Aston-Jones et al. 1982; Osmanovic and Shefner 1994; Strahlendorf and Strahlendorf 1983; Verbanck et al. 1990). Even without direct changes in basal activity, LC signaling of salient sensory information is disrupted by low dose ethanol (1 g/kg). Acute ethanol delays, reduces the magnitude of, and slows LC-NE conduction velocity during LC signaling of sensory

information (Aston-Jones et al. 1982). This difference between, on the one hand, suppression of NE unit activity or responsiveness and, on the other hand, evidence of neuronal activation using metabolite and c-Fos measures, has yet to be resolved. One potential explanation worth pursuing, however, comes from in vitro evidence for rebound activation in LC-NE neurons after acute ethanol washout which has been seen in multiple studies after acute suppression, or complete inhibition of firing by ethanol (Shefner and Tabakoff 1985; Verbanck et al. 1990).

There is additional evidence that environmental history may alter NE responses to acute alcohol, which has implications for interpretation of the above findings. Karkhanis et al. (2014, 2015) used microdialysis to measure NE release in the basolateral amygdala and NAc after low doses of acute ethanol (1 or 2 g/kg). No changes in NE release after alcohol were identified in group housed animals, but there was a significant enhancement of NE release within animals that had a history of social isolation. Just as environment might alter neural plasticity that contributes to changes in NE response to acute alcohol, chronic alcohol consumption also appears to alter the homeostatic balance within NE circuitry.

As noted above, NE metabolite levels in the CSF of alcohol-dependent subjects are higher compared to control subjects following acute ethanol administration (Borg et al. 1981). In dependent animals receiving daily ethanol gavage, NE metabolite levels remain high while intoxicated and through to withdrawal, indicating chronic increases in NE signaling (Karoum et al. 1976). Repeated alcohol administration has also been shown to sensitize NE neurons to release larger amounts of NE (Lanteri et al. 2008). Chronic alcohol differentially induces c-Fos signaling in the NTS (Ryabinin et al. 1997) of males and LC of females but not males (Chang et al. 1995; Retson et al. 2015) indicating some region-specific adaptations in NE signaling with chronic alcohol. However, withdrawal from chronic alcohol ubiquitously activates NE signaling across regions (Vilpoux et al. 2009).

Chronic alcohol use has long been associated with disruption of the HPA axis, although central NE is not necessarily a direct component (Richardson et al. 2008). As noted above, NE sources such as LC and NTS provide critical input to central HPA nuclei including the paraventricular nucleus of the hypothalamus (PVN) as well as sites in the amygdala and extended amygdala (Moore and Bloom 1979). β-NE signaling within PVN is critical to ACTH production after ethanol administration (Selvage 2012). Alterations in NE signaling to HPA regions, combined with reciprocal connections between NE nuclei and CRF neurons that are activated after chronic ethanol in the central amygdala and other regions, generate feed-forward circuits for persisting NE dysfunction after chronic alcohol (Retson et al. 2016). Evidence for allostatic changes in NE signaling after chronic alcohol exposure and withdrawal indicates NE dysfunction as a key aspect of the negative affective state and a component driving relapse after abstinence (Koob 2014).

In summary, there is a wealth of studies demonstrating that both acute and chronic alcohol use has an impact on NE neuron function and NE release (see Table 1). Additionally, several peptides, such as NPY and galanin, which are expressed by NE neurons (and other populations) are known to be altered by alcohol exposure (Barson and Leibowitz 2016; Gilpin and Roberto 2012). The impact of

Table 1 Summary of the impact of ethanol on NE neuronal function

Change	Region	Measurement	Species	References
NE neuron activity				
Acute ethanol				
Decrease	LC	Electrophysiology (in vivo)	Rodent	Aston-Jones et al. (1982), Strahlendorf and Strahlendorf (1983), and Verbanck et al. (1990)
Decrease	LC	Electrophysiology (ex vivo)	Rodent	Osmanovic and Shefner (1994), Shefner and Tabakoff (1985), and Verbanck et al. (1990)
Rebound increase	LC	Electrophysiology (ex vivo)	Rodent	Shefner and Tabakoff (1985) and Verbanck et al. (1990)
Increase	LC, NTS	c-Fos	Rodent	Aimino et al. (2017), Chang et al. (1995), Kolodziejska-Akiyama et al. (2005), Lee et al. (2011), Ryabinin et al. (1997), and Thiele et al. (1997, 2000)
Chronic ethanol				
Increase	LC	c-Fos	Rodent	Males (Knapp et al. 1998; Putzke et al. 1996; Ryabinin et al. 1997) females but not males (Retson et al. 2015)
Decrease	LC	c-Fos	Rodent	Males (Rodberg et al. 2017)
NE release/measurement				
Acute ethanol				
Increase	CSF/ plasma	NE, metabolites (MHPG)	Human	Borg et al. (1981) and Howes and Reid (1985)
Increase	Whole brain	Metabolites (DHPG, VMA)	Rodent	Alari et al. (1987b), Corrodi et al. (1966), Karoum et al. (1976), and Murphy et al. (1983)
Decrease	Whole brain	NE	Rodent	Alari et al. (1987a) and Murphy et al. (1983)
Chronic ethanol				
Increase	CSF	Metabolites (MHPG)	Human	Borg et al. (1981)
Increase	Whole brain	Metabolites (DHPG, VMA)	Rodent	Karoum et al. (1976)
Increase	PFC	Evoked extracellular NE	Rodent	Lanteri et al. (2008)

As discussed in the text above, there has been longstanding interest in understanding how ethanol alters noradrenergic activity, and norepinephrine release/turnover. Some key findings are summarized in this table

BLA basolateral amygdala, *CSF* cerebrospinal fluid, *DHPG* 3,4-dihydroxyphenylglycol, *MHPG* 3-methoxy-4-hydroxy-phenylglycol, *NAc* nucleus accumbens, *LC* locus coeruleus, *NTS* nucleus tractus solitarius, *PFC* prefrontal cortex, *VMA* vanillomandelic acid

co-transmitter/peptide release within NE circuits remains understudied in general. Most studies on these systems to date have focused on receptor signaling, and the source of these relevant co-transmitters/peptides, including whether or not they are

originating in NE neurons, remains to be determined. There are also results that appear to conflict – the differences between decreased acute effects on NE neuron activity vs. increased c-Fos and NE release, for example. These differences may stem from experimental differences such as time points of intoxication and withdrawal, all of which have profound but potentially differential effects on NE systems. Future work dissecting the effects of acute vs. chronic alcohol at different stages of administration and withdrawal will help specify the precise effects of alcohol on NE neuron function and plasticity.

3.2 Effects of Noradrenergic Receptor Modulation on Alcohol-Related Behaviors and Neural Systems

Manipulation of noradrenergic signaling has provided some of the strongest evidence for a functional role of NE in alcohol-related behaviors and has demonstrated not only a mechanism for NE in stress-associated alcohol effects, but in positive motivational aspects of alcohol use as well (Table 2). Early studies using DBH inhibitors showed attenuation of voluntary ethanol consumption (Amit et al. 1977). More recently DBH knockout mice that are incapable of producing central NE have been shown to have a number of relevant phenotypes including reduced ethanol consumption in males but not females, and increased ethanol-related hypothermia and sedation in both sexes (Weinshenker et al. 2000). DBH knockout mice are hyperdopaminergic and release DA from NE terminals, suggesting the DA signaling through NE neurons may be a potential mechanism for these findings. However disruptions of DA signaling through lesioning of accumbens DA inputs do not impact voluntary ethanol intake (Rassnick et al. 1993). Lesions of ascending NE tracts can generate increases (Kiianmaa and Attila 1979; Kiianmaa et al. 1975) or decreases in ethanol intake (Brown and Amit 1977; Corcoran et al. 1983).

Whether NE-related changes in ethanol consumption are representative of reduced stress-driven drinking or reduced ethanol reward remain unclear. However, strong evidence for rewarding components of NE in acute alcohol comes from a selective NE deafferentation of medial prefrontal cortex (mPFC) in mice which prevents ethanol-related conditioned place preference and reduces ethanol consumption (Ventura et al. 2006). This reinforces that the complexity of NE in alcohol-related behaviors is due in part to the broad efferent networks of NE and complex interactions at different targets.

A number of studies investigating the role of NE transmission in dependent subjects indicate a role for NE in pathological allostasis that is further exacerbated during withdrawal (Becker 2012; Koob 2014). NE-targeted therapies have demonstrated efficacy in improving symptoms associated with sympathomimetic overdrive and NE overactivation during withdrawal from chronic alcohol (Hawley et al. 1994; Rasmussen et al. 2006). Polymorphisms in noradrenergic reuptake transporters or α2 receptors, both mechanisms for terminating NE signaling, have been associated with familial history of, or individuals with, alcohol use disorders (Clarke et al. 2012). Studies in rodents and humans have shown value in the use of

Table 2 Summary of the impact NE manipulations on ethanol-related behavior

Direct NE disruption				
Manipulation	**Measurement**	**Effect on ethanol-related behavior**	**Species**	**References**
DBH knock-out	Ethanol intake	Decreased consumption	Rodent	Males but not females (Weinshenker et al. 2000)
DBH inhibition	Ethanol intake	Decreased consumption	Rodent	Brown et al. (1977)
DNAB lesion	Ethanol intake	Increased consumption	Rodent	Kiianmaa et al. (1975)
DNAB lesion	Ethanol intake and initiation	Decreased consumption/ initiation	Rodent	Brown and Amit (1977) and Corcoran et al. (1983)
NE lesion in PFC	Ethanol intake and CPP	Decreased consumption/ preference	Rodent	Ventura et al. (2006)
LC lesion	Withdrawal symptoms	Decrease	Rodent	Kostowski and Trzaskowska (1980)
NE receptor disruption				
Target/ manipulation (compound)	**Measurement**	**Effect on ethanol-related behavior**	**Species**	**References**
α2 – Gi coupled				
Agonist (lofexidine, guanfacine)	Ethanol SA, cue/ stress-induced reinstatement	Decreased ethanol seeking	Rodent	Fredriksson et al. (2015), Le et al. (2005), and Riga et al. (2014)
Agonist (clonidine, guanfacine)	Ethanol intake	Decreased consumption	Rodent	Fredriksson et al. (2015), Opitz (1990), and Rasmussen et al. (2014a)
Agonist (clonidine)	Withdrawal symptoms	Decrease acute withdrawal	Rodent	Kostowski and Trzaskowska (1980)
α1 – Gq coupled				
Inverse agonist (prazosin)	Cue/stress-induced craving	Decreased ethanol craving	Human	Fox et al. (2012)
Inverse agonist (prazosin)	Ethanol intake	Decreased consumption	Human	Simpson et al. (2009, 2015)
Inverse agonist (prazosin)	Ethanol intake and initiation	Decreased consumption	Rodent	Froehlich et al. (2013, 2015) and Skelly and Weiner (2014)
Inverse agonist (prazosin)	Ethanol SA, cue/ stress-induced reinstatement	Decreased ethanol seeking	Rodent	Funk et al. (2016), Verplaetse et al. (2012), and Walker et al. (2008)
Antagonist (doxazosin)	Ethanol intake	Decreased consumption	Rodent	O'Neil et al. (2013)
Antagonist (doxazosin)	Cue/stress-induced ethanol reinstatement	Decreased ethanol seeking	Rodent	Funk et al. (2016)

(continued)

Table 2 (continued)

NE receptor disruption				
β – Gs coupled				
Antagonist (propranolol)	Withdrawal symptoms	Decrease withdrawal	Human	Carlsson (1976) and Sellers et al. (1977)
Antagonist (propranolol)	Ethanol SA	Decreased ethanol seeking	Rodent	Gilpin and Koob (2010)
Antagonist (propranolol)	Ethanol intake	Decreased consumption	Rodent	Andreas et al. (1983)
α1 and β antagonist cocktail				
Prazosin + propranolol	Ethanol intake	Decreased consumption	Rodent	Rasmussen et al. (2014b)

NE-targeted manipulations have been shown to have a number of effects on ethanol-related behavior. The vast majority of interventions have targeted reductions in NE transmission/signaling and a summary of some key effects are included in this table
CPP conditioned place preference, *DBH* dopamine-β-hydroxylase, *DNAB* dorsal noradrenergic bundle, *PFC* prefrontal cortex, *SA* self-administration (operant)

Gi-coupled α2-adrenergic agonists (which activate postsynaptic and autoreceptors) as adjuncts for ameliorating ethanol withdrawal (Kostowski and Trzaskowska 1980; reviewed by Muzyk et al. 2011).

Preclinical evidence indicates an effect of NE pharmacotherapy both during acute withdrawal and in the maintenance of abstinence after chronic ethanol. α2 agonists have shown further application in reducing operant self-administration of ethanol and reduce stress-induced reinstatement of ethanol seeking (Le et al. 2005). In alcohol preferring strains of P and AA rats, α2 agonists reduce voluntary alcohol intake acutely and for several days after repeated administration (Opitz 1990; Rasmussen et al. 2014a). These effects are likely mediated by overall reductions in NE signaling due to presynaptic modulation as postsynaptic NE antagonists produce similar effects. Guanfacine, an α2a agonist known for cognitive enhancing effects and currently being explored for treating ADHD (Ramos and Arnsten 2007), has been tested in rat models of drinking, producing decreased alcohol intake in high-drinking rats (Fredriksson et al. 2015) and in rats with elevated drinking resulting from social defeat stress (Riga et al. 2014). These results indicate a complex, but potentially important role for α2 signaling in alcohol motivation and AUD. However, these agonist studies must be interpreted with caution, as α2 agonists have known sedative properties via Gi-mediated inhibition of arousal circuits (Aoki et al. 1994) that may reduce a variety of volitional behaviors, particularly after systemic administration.

Chronic alcohol has been shown to disrupt α1-mediated NE signaling in the extended amygdala (McElligott et al. 2010), similar dysfunction likely occurs at other postsynaptic targets with extensive α1 receptors including hypothalamus, amygdala, prefrontal cortex, and VTA (Domyancic and Morilak 1997; Sands and Morilak 1999). Prazosin, an α1 NE inverse agonist, is a sympatholytic compound

which is FDA approved to treat hypertension and has been well explored in relation to alcohol consumption. It has been shown to dose dependently reduce operant ethanol seeking in dependent animals (Walker et al. 2008), seeking in P rats (Verplaetse et al. 2012), and relapse in P rats (Froehlich et al. 2015), and it can also delay initiation of drinking in P rats (Froehlich et al. 2013). Prazosin also reduces anxiety-like behavior after chronic alcohol exposure and ongoing ethanol consumption in rats (Rasmussen et al. 2017; Skelly and Weiner 2014), and decreases sensitization to chronic alcohol administration in mice (Kim and Souza-Formigoni 2013). Prazosin crosses the blood–brain barrier and is available as a clinical antihypertensive agent. Off-label studies have provided translational support for prazosin in the treatment of AUD. Prazosin has been shown to reduce stress- and cue-induced alcohol craving in abstinent individuals with AUD (Fox et al. 2012), as well as to reduce alcohol consumption and increase alcohol free days in treatment seeking individuals with AUD and those with AUD and comorbid PTSD (Simpson et al. 2009, 2015). Doxazosin, a long-lasting α1 receptor antagonist, has shown preclinical efficacy in reducing alcohol consumption and yohimbine-induced reinstatement in alcohol preferring P rats (Funk et al. 2016; O'Neil et al. 2013). α1-meditated treatments such as prazosin and doxazosin may also come with potential side effects, such as orthostatic hypertension, and at high doses drowsiness, which may either limit their usefulness or patient compliance (see clinical efficacy review in this volume, Litten et al. 2018). However, both preclinical and human tests of α1 antagonists appear to be efficacious, as described above, suggesting that refinement of α1-associated therapy may be a worthwhile pursuit.

Signaling through the β adrenergic receptor influences stress responses, indicating a potential mechanism to explain AUD-associated stress and anxiety (Do Monte et al. 2008; Giustino et al. 2016; Gorman and Dunn 1993; Steenen et al. 2016). However, the contributions of this system to AUD have been less thoroughly explored. Early studies suggested a beneficial role of propranolol, a nonselective β adrenergic antagonist, on decreasing withdrawal symptoms such as elevated anxiety and potentially reducing drinking in human alcoholic patients (Carlsson 1976; Sellers et al. 1977). Propranolol treatment also decreased alcohol preference in mice (Andreas et al. 1983). In rats, propranolol reduced operant self-administration of and motivation for alcohol in dependent animals at low doses and reduced moderate ethanol consumption in nondependent animals at high doses (Gilpin and Koob 2010). Combined α1 and β adrenergic treatment with prazosin and propranolol is more effective at reducing alcohol consumption than either drug alone in rats (Rasmussen et al. 2014b), indicating that such a combination treatment may be useful in patients. However, this promising approach has not yet been investigated clinically. As noted with respect to α1-related treatments above, potential NE-related pharmacotherapies targeting β-adrenergic receptors must be considered with caution. Drugs targeting these receptors have potent hypotensive effects (Musini et al. 2017) which may limit their implementation and relevant dosing.

As is clear from studies involving manipulation of NE receptor signaling, there is a prominent role for this pathway in both human and animal models of alcohol use

(Table 2). The potent effects of α1 receptor blockade in animals and the promising impacts on human patients indicate that this may be a key mechanism in regulating aspects of AUD. However, modulation at α2 or β receptors may play an equally potent role, and future therapies may benefit from combinations of receptor targeting. Although NE receptor pharmacotherapy appears to be particularly promising for AUD treatment, refinements are still required in order to develop therapies that are specific to dependence symptoms, including increased alcohol motivation, increased stress, and potentially cognitive disruptions impairing decision making. One possibility is that the AUD syndrome is a result of globally disrupted NE signaling, arguing that broad NE-associated treatments will be maximally efficacious. Alternately, only some aspects of the NE system may be disrupted in AUD, or AUD subtypes, suggesting that future pharmacotherapy treatment should be refined. One way to refine future treatments, and a key action item for future research, is to determine functional consequences of the intersection between NE receptor subtypes and downstream neural systems influenced. Specific symptoms of AUD may result from disrupted NE signaling specifically in the BNST or amygdala or PFC, for example, but not in other areas. Individualized, highly specific treatment for subtypes of AUD may stem from characterizing precise interactions between NE release and NE-regulated neural networks. In addition to understanding specific circuits influenced by AUD-associated disruptions in NE release, future NE-related treatments may benefit from an understanding of interactions between NE signaling and other neuromodulatory or peptidergic pathways. Given the diverse set of systems associated with alcohol use and AUD, optimal treatments for alcohol-associated disorders will likely benefit from targeting multiple systems. One possibility is that these systems interact sequentially, such as in the proposal that the main motivational impact of NE is via the regulation of DA release, and the observation that neuropeptides such as DYN or CRF impact signaling in the LC, for example (Tjoumakaris et al. 2003). Alternately, these systems may influence alcohol use in a simultaneous, distributed, and potentially independent fashion, increasing the diversity of AUD subtypes depending on combinations of systems affected. Thus, in addition to specifying the precise nature of NE impact on alcohol use, potential treatment research will benefit from understanding the interaction (or lack thereof) between NE and the numerous other neural systems disrupted in AUD (Becker 2012; Koob 2014).

4 Summary: Norepinephrine in the Treatment of AUD

As discussed in the sections above, NE signaling exhibits a substantial degree of influence over alcohol use and its disruption contributes to AUD. Although its role in stress and anxiety is well documented, NE also appears to have an important role in the rewarding aspects of alcohol use. The NE system is also critical in a number of other behaviors and functions, in particular cognitive functions such as attention, memory, and decision making, as described above. Each of these elements of NE regulation contribute both to the role of NE signaling in regulated, nondependent,

alcohol seeking and use, and in AUD symptoms resultant from chronic alcohol exposure. Given the prominent dysphoric disruptions that are a consequence of chronic alcohol use, elevated NE associated with (or even producing) increased stress in AUD may enhance motivation for alcohol in order to transiently relieve this negative hedonic state (Koob 2014). At the same time, disrupted NE control over cognitive functions such as response inhibition, behavioral flexibility, attention, and memory may result in compromised mechanisms that would normally allow individuals to regulate alcohol seeking behaviors. Pharmacotherapeutics specifically targeting the NE system, therefore, have the potential to ameliorate multiple key symptoms of AUD: motivation for alcohol, stress, and cognitive disruption. Supporting this hypothesis, initial results in human patients treated with prazosin, for example, appear promising. More specifically tailored treatments resulting from future research into the exact mechanisms of NE receptor subtypes and their interaction with other brain systems will enhance the effectiveness and selectivity of targeting the NE system. Additional issues remain to be explored at both basic and clinical levels, such as the interaction between NE signaling and other neuromodulators and neuropeptides, clear sex differences in NE function in both humans and nonhuman models, and issues associated with individual heterogeneity in AUD symptoms in which some may be more NE-associated than others. Regardless, work to date has demonstrated that the NE system is both fundamentally involved in alcohol seeking behaviors and is disrupted following chronic alcohol and withdrawals in AUD and research for NE-targeted pharmacotherapies has strong potential for positive treatment outcomes.

Acknowledgments This work was supported by NIH grants AA024571 (EMV/DEM) and AA025481 (DEM).

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Part II

Ion Channels

Voltage-Sensitive Calcium Channels in the Brain: Relevance to Alcohol Intoxication and Withdrawal

Prosper N'Gouemo

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Abstract

Voltage-sensitive Ca^{2+} (Ca_V) channels are the primary route of depolarization-induced Ca^{2+} entry in neurons and other excitable cells, leading to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The resulting increase in $[Ca^{2+}]_i$ activates a wide range of Ca^{2+}-dependent processes in neurons, including neurotransmitter release, gene transcription, activation of Ca^{2+}-dependent enzymes, and activation of certain K^+ channels and chloride channels. In addition to their key roles under physiological conditions, Ca_V channels are also an important target of alcohol, and alcohol-induced changes in Ca^{2+} signaling can disturb neuronal homeostasis, Ca^{2+}-mediated gene transcription, and the function of neuronal circuits, leading to various neurological and/or neuropsychiatric symptoms and disorders, including alcohol withdrawal induced–seizures and alcoholism.

P. N'Gouemo (✉)
Department of Pediatrics, Georgetown University Medical Center, Washington, DC, USA
e-mail: pn@georgetown.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_93

Keywords
Alcohol exposure · Alcohol intoxication · Alcohol withdrawal seizures · Calcium signaling

1 Introduction

In neurons, voltage-sensitive Ca^{2+} (Ca_V) channels serve as the primary route of Ca^{2+} entry in response to membrane depolarization, driving a localized increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The driving force for Ca^{2+} entry arises from the steep electrochemical gradient maintained between extracellular and intracellular Ca^{2+} concentrations, which are typically on the order of 1 mM and 100 nM, respectively; thus Ca^{2+} entry can change membrane potential and can therefore affect neuronal excitability. In neurons, low $[Ca^{2+}]_i$ is maintained by a variety of mechanisms and processes, including Ca^{2+} efflux via a Na^+/Ca^{2+} exchange protein and a Ca^{2+}-ATPase located at the plasma membrane, as well as the sequestration of intracellular Ca^{2+} into in Ca^{2+} stores (e.g., via the sarco-endoplasmic reticular ATPase pump) or by Ca^{2+}-buffering proteins (Berridge 2012).

The Ca_V-mediated localized increase in $[Ca^{2+}]_i$ in neurons activates a variety of downstream processes, including Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+}-gated Ca^{2+} stores, activation of Ca^{2+}-activated K^+ channels, Ca^{2+}-activated chloride channels and Ca^{2+}-dependent enzymes, and other Ca^{2+}-dependent processes such as gene transcription and neurotransmitter release. In addition, Ca^{2+} entry following relatively mild membrane depolarization (e.g., depolarization induced by activation of *N*-methyl-D-aspartate receptors) can give rise to low-threshold Ca^{2+} spikes, which can further depolarize the plasma membrane, causing voltage-gated Na^+ channels to open and initiating the repetitive firing of action potentials (Cain and Snutch 2010). Thus, Ca_V channels play a wide range of important roles under both physiological and pathophysiological conditions, including a variety of diseases associated with neuronal excitability. In the central nervous system (CNS), Ca_V channels are also an important molecular target for alcohol, and changes in neuronal Ca^{2+} signaling induced by alcohol exposure and subsequent withdrawal can lead to alcoholism and alcohol withdrawal–induced seizures, (AWSs).

2 Structure, Diversity, and Localization of Voltage-Sensitive Ca^{2+} Channels in the CNS

2.1 Structure and Diversity of Ca_V Channels

Ca_V channels are large protein complexes comprised of a pore-forming α1 subunit and up to three auxiliary β, α2/δ, and γ subunits (Simms and Zamponi 2014). In addition to providing the pore through which Ca^{2+} flows, the α1 subunit of Ca_V channels also confers the channel's electrophysiological and pharmacological

properties; in contrast, the auxiliary subunits modulate the channel's biophysical properties and regulate the channel's trafficking to the plasma membrane. In human, nine distinct genes encode the α_1 subunits (designated α_{1A} through α_{1I}), all of which are expressed in the CNS (Simms and Zamponi 2014). Based on their responsiveness to changes in membrane potential, these nine Ca_V channels are broadly classified as either low voltage–activated (LVA, comprising the Ca_V3 family) channels or high voltage–activated (HVA, which include the Ca_V1 and Ca_V2 families) channels. Activation of LVA channels and HVA channels produced transient and sustained currents, respectively.

HVA Ca_V channels have both distinct and overlapping voltage dependence and kinetics, making it difficult to differentiate HVA Ca_V currents based solely on their biophysical properties. Fortunately, however, HVA Ca_V channels have unique pharmacological profiles, which have been used to confirm the heterogeneity of the channels expressed in the CNS. Moreover, based largely on their sensitivity to various Ca_V channel blockers, HVA Ca_V channels currents have been further classified into the following five types: L-type $Ca_V1.2$ (α_{1C}), L-type $Ca_V1.3$ (α_{1D}), N-type $Ca_V2.2$ (α_{1B}), P/Q-type Cav2.1 (α_{1A}), and R-type $Ca_V2.3$ (α_{1E}) channels, encoded by the *CACNA1C*, *CANA1D*, *CANA1B*, *CANA1A*, and *CACNA1E* genes, respectively (Ertel et al. 2000; Randall and Tsien 1995). In the CNS, P/Q-type Cav2.1 channels can give rise to both P-type and Q-type currents; this distinction is likely due to a combination of factors, including the Ca_V-β subunit and/or alternative splicing of the *CACNA1A* gene that encodes the channels (Richards et al. 2007).

Molecular analyses revealed that the LVA family of Ca_V channels consists of three distinct α_1 pore-forming subunits, namely $Ca_V3.1$ (α_{1G}), $Ca_V3.2$ (α_{1H}), and $Ca_V3.3$ (α_{1I}), encoded by the *CACNA1G*, *CANA1H*, and *CACNA1I* genes, respectively (Cribbs et al. 1998; Lee et al. 1999; Perez-Reyes et al. 1998). Interestingly, unlike HVA Ca_V channels, the α1 subunit of LVA Ca_V channels does not require auxiliary subunits to form a fully functional channel, although LVA Ca_V channels can be regulated by auxiliary subunits (Klöckner et al. 1999). Finally, the three genes that encode the $Ca_V3.x$ subunits can undergo alternative splicing, giving rise to a wide diversity of functional LVA Ca_V channels (Swayne and Bourinet 2008). The Ca_V-α_1 subunit is comprised of four transmembrane domains, which are connected by cytoplasmic linkers (Simms and Zamponi 2014; Turner and Zamponi 2014). The N and C termini are located in the cytoplasmic side and they contained important sites for protein–protein interactions such as with G-protein and protein kinases (Simms and Zamponi 2014; Turner and Zamponi 2014). Interestingly, phosphorylation by PKA or PKC alters the voltage dependence and kinetics of Ca_V currents (Gray and Johnston 1987; Nagao and Adachi-Akahane 2001; Sculptoreanu et al. 1993; Stea et al. 1995).

2.2 Localization and Function HVA Ca_V1 Channels

Although L-type $Ca_V1.x$ channels are expressed widely throughout brain, each channel subtype has a unique cellular and subcellular distribution. For example, L-type $Ca_V1.3$ channels are distributed relatively evenly, whereas L-type $Ca_V1.2$ channels are localized in clusters (Hell et al. 1993; Tippens et al. 2008). Moreover, L-type $Ca_V1.2$ and $Ca_V1.3$ channels are located predominantly on the cell soma (where they regulated depolarization and Ca^{2+}-dependent pathways that control gene expression), proximal dendrites, and in some interneurons in the olfactory bulb, cerebral cortex (pyramidal neurons), hippocampus (pyramidal neurons in the CA1–CA3 areas), dentate gyrus (granule neurons), amygdala, inferior colliculus, cerebellum (granule layer, molecular layer, Purkinje cells), and spinal cord (Hell et al. 1993). Unlike L-type $Ca_V1.3$ channels, $Ca_V1.2$ channels are expressed in astrocytes in the CA3 area of the hippocampus (Tippens et al. 2008; Westenbroek et al. 1990). The distribution of $Ca_V1.2$ and $Ca_V1.3$ channels throughout the CNS has been confirmed by RT-PCR analysis, which shows that the levels of *CACNA1C* and *CACNA1D* mRNA matches the protein levels of Ca_V-α1C and Ca_V-α1D subunits, respectively (Sinnegger-Brauns et al. 2009; Schlick et al. 2010). In the striatum, *CACNA1C* and *CACNA1D* mRNA are co-expressed in medium-sized spiny neurons (Olson et al. 2005). Interestingly, L-type $Ca_V1.3a$ (but not $Ca_V1.3b$) isoform co-localizes with Shank protein and the synaptic protein PSD-95 in medium spiny neurons at excitatory synapses (Olson et al. 2005). In the CNS, approximately 80% and 20% of L-type Ca_V1 channels are $Ca_V1.2$ and $Ca_V1.3$ channels, respectively (Hell et al. 1993; Sinnegger-Brauns et al. 2009). With respect to function, evidence suggests that L-type $Ca_V1.3$ channels activate with less depolarization and inactivate more slowly than $Ca_V1.2$ channels (Koschak et al. 2001; Xu and Lipscombe 2001). Given their unique set of biophysical properties, L-type $Ca_V1.3$ channels likely play an important role in controlling Ca^{2+}-dependent firing; moreover, L-type $Ca_V1.3$ channels help sustain Ca^{2+} influx at membrane potentials at which $Ca_V1.2$ channels are closed.

$Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$ channels (i.e., P/Q-type, N-type, and R-type, respectively) are also expressed throughout the CNS. P/Q-type $Ca_V2.1$ channels are primarily concentrated in presynaptic terminals and dendritic shafts, N-type $Ca_V2.2$ are found mainly in dendrites and some cell bodies of neurons, and R-type $Ca_V2.3$ channels are found mainly in the cell soma in most sites with variable expression in dendrites (Westenbroek et al. 1992, 1995; Yokoyama et al. 1995). These Ca_V channels are found primarily in the olfactory bulb, cerebral cortex (pyramidal neurons), striatum (medium-sized spiny neurons), amygdala, hippocampus (pyramidal neurons in CA1–CA3 areas), dentate gyrus (granule neurons), thalamus, globus pallidus, hypothalamus, inferior colliculus, and cerebellum (Purkinje cells) (Hillman et al. 1991; Westenbroek et al. 1992, 1995; Volsen et al. 1995; Yokoyama et al. 1995; Day et al. 1996; Xu et al. 2010). In the cortex and hippocampus, there is barely detection of R-type $Ca_V2.3$ channels in proximal dendrites, while other structures such as olfactory bulb, amygdala, and cerebellum have intense expression of these channels in the dendrites, the

prominent sites of Ca^{2+} entry, causing transient increase in cytosolic Ca^{2+}. Molecular and biochemical analyses have confirmed that mRNA levels match the corresponding protein for $Ca_V2.1(\alpha_{1A})$, $Ca_V2.2(\alpha_{1B})$, and $Ca_V2.3$ (α_{1E}) (Mori et al. 1991; Soong et al. 1993; Day et al. 1996; Ludwig et al. 1997; Schlick et al. 2010).

At synaptic terminal, the rapid release of neurotransmitters requires tight coupling between presynaptic $Ca_V2.x$ channels to the release machinery. In addition to regulating vesicle fusion, members of the $Ca_V2.x$ channels also control neuronal excitability. For example, P/Q-type $Ca_V2.1$ and N-type $Ca_V2.2$ channels interact both physically and functionally with large-conductance, Ca^{2+}-activated K^+ channels, providing the Ca^{2+} influx needed to activate these channels (Faber and Sah 2003; Berkefeld et al. 2010); thus, P/Q-type $Ca_V2.1$ and N-type $Ca_V2.2$ channels control neuronal excitability by regulating K^+ conductances.

2.3 Localization and Function LVA Ca_V3 Channels

Like HVA Ca_V channels, LVA Ca_V3 channels are also distributed throughout the CNS; however, their expression is restricted to the cell body and dendrites of neurons primarily in the olfactory bulb (granule layer), cerebral cortex (pyramidal neurons, GABAergic interneurons), striatum, amygdala, hippocampus (CA1–CA3 pyramidal neurons), dentate gyrus (granule cells), thalamus (large neurons, GABAergic interneurons), substantia nigra, inferior colliculus, superior colliculus, inferior olive, cerebellum (granule layer, molecular layer, Purkinje cells), and spinal cord (Craig et al. 1999; Talley et al. 1999; Yunker et al. 2003; McKay et al. 2006; Kovács et al. 2010; Liu et al. 2011; Kanyshkova et al. 2014).

As discussed above, LVA Ca_V3 channels are activated upon weak depolarization and carry depolarizing currents; therefore, similar to L-type $Ca_V1.3$ channels, LVA $Ca_V3.x$ channels also play an important role in controlling neuronal excitability. LVA $Ca_V3.x$ channels also inactivate at a fast rate. Thus, a combination of low threshold of activation with fast inactivation kinetics results in transient Ca^{2+} influx, giving rise to the so-called "low-threshold Ca^{2+} potentials," which initiate the burst-firing process (Cain and Snutch 2010; Contreras 2006; Jahnsen and Llinas 1984; Lee et al. 2003; Yazdi et al. 2007; Xu and Clancy 2008). The burst-firing mode in the CNS contributes to the generation of physiological events such as sleep spindles, and pathological conditions such as epileptic seizures (Cain and Snutch 2010, 2012). In addition, LVA $Ca_V3.x$ channels generate a so-called "window current" near the neuron's resting membrane potential, thereby regulating Ca^{2+} homeostasis (Dreyfus et al. 2010). In the CNS, LVA $Ca_V3.x$ channels are also associated both with voltage-gated K^+ channels and with Ca^{2+}-activated K^+ channels (Anderson et al. 2010; Rehak et al. 2013), giving LVA $Ca_V3.x$ channels the ability to activated K^+ channels and regulate neuronal firing.

3 Effects of Acute Alcohol Exposure on the Expression and Function of Ca_V Channels

Oakes and Pozos (1982a, b) reported that alcohol exposure decreased Ca_V currents (and voltage-gated K^+ currents but not voltage-gated Na^+ currents) in dorsal root ganglia neurons. This effect was not associated with change in the resting membrane potential and spike amplitude. However, the duration of the action potential (AP) was decreased, and AP threshold was increased (Oakes and Pozos 1982a, b). A large body of experimental evidence indicates that acute alcohol exposure suppresses K^+ depolarization–induced and AP–evoked Ca^{2+} transients in several CNS neurons including inferior colliculus, cerebellar, and hippocampal neurons (Gruol et al. 1997; Mah et al. 2011; Morton and Valenzuela 2016; our unpublished data). Consistent with these findings, we found that acute alcohol exposure inhibits the current carried by HVA Ca_V channels in inferior colliculus neurons (our unpublished data). Furthermore, acute alcohol exposure suppresses currents through L-type $Ca_V1.x$ channels at neurohypophysial terminals, in supraoptic neurons, and hippocampal neurons (Wang et al. 1991, 1994; Widmer et al. 1998; Zucca and Valenzuela 2010). On the other hand, P-type $Ca_V2.1$ channels in Purkinje cells are unaffected by acute alcohol exposure (Hall et al. 1994). Thus, in the CNS, L-type $Ca_V1.x$ channels appear to be particularly sensitive to the acute effects of alcohol exposure.

Interestingly, LVA $Ca_V3.x$ channels are also an important target for alcohol. For example, acutely exposing rodent thalamic neurons to a low or high alcohol concentration increases or decreases, respectively, LVA $Ca_V3.x$ currents (Mu et al. 2003; Joksovic et al. 2005). Furthermore, the inhibitory effect of alcohol on LVA $Ca_V3.x$ currents appears to be mediated by protein kinase C (Shan et al. 2013). In contrast, acute exposure to either low or high alcohol concentration inhibits LVA $Ca_V3.x$ currents in the inferior olive in primates (Welsh et al. 2011). Thus, the increase in LVA $Ca_V3.x$ currents in response to low alcohol concentration in rodents – but not in primates – suggests species-specific differences in the underlying mechanisms.

The inhibition of HVA Ca_V channels and LVA Ca_V channels (Fig. 1), and downstream Ca^{2+}-related signaling following acute alcohol exposure suggests that this mechanism may induce a compensatory upregulation of HVA Ca_V channels and LVA Ca_V channels during chronic alcohol intoxication; this upregulation would be masked by the inhibitory effect alcohol, but would then be revealed during alcohol withdrawal.

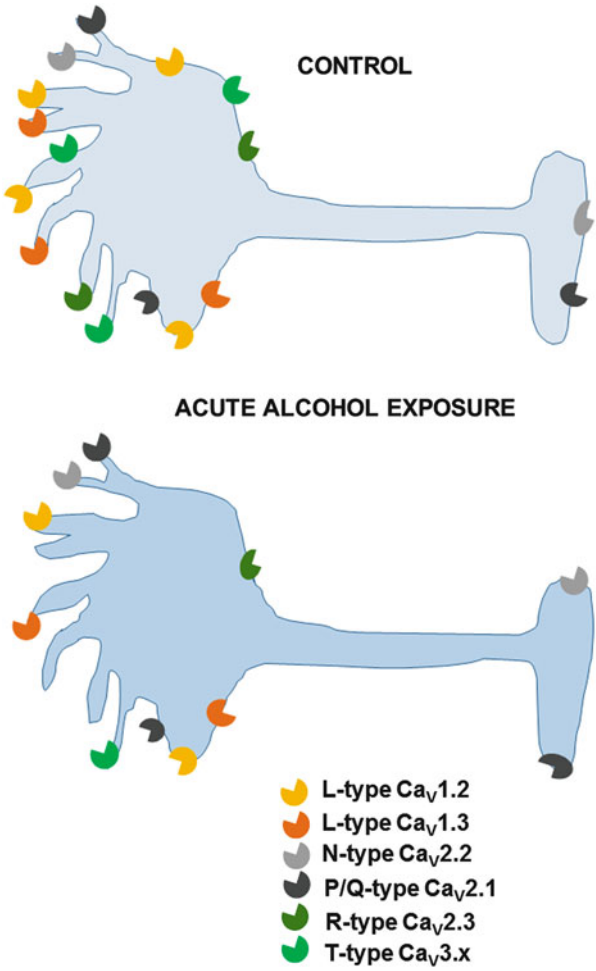

Fig. 1 Acute alcohol intoxication downregulated Ca_V channels in the brain. In normal conditions, the expression of LVA and HVA Ca_V channels is tightly regulated whereas following acute alcohol intoxication, a dysregulation occurs leading to a downregulation of LVA and HVA Ca_V channels (L-type $Ca_V1.x$)

4 Effects of Chronic Alcohol Exposure on the Expression and Function of Ca_V Channels

Several lines of evidence indicate that chronic alcohol exposure alters Ca^{2+} signaling in the CNS. For example, chronic alcohol exposure increases AP–evoked Ca^{2+} transients in hippocampal neurons (Mulholland et al. 2015), possibly by upregulating of Ca_V channels. Consistent with this notion, P-type $Ca_V2.1$ current is increased in the cerebellum during chronic alcohol exposure (Gruol and Parsons 1994). On the other hand, chronic alcohol intoxication by inhalation did not alter the protein levels of P/Q-type $Ca_V2.1$ (α_{1A}) protein levels in cortical neurons (Katsura et al. 2005). Similarly, the protein levels of the P/Q-type α_{1A} subunit were

unchanged in the central nucleus of the amygdala following chronic intermittent alcohol exposure (Varodayan et al. 2017a). Increased protein levels of L-type $Ca_V1.3$ (α_{1D}) channels were measured in cortical neurons in mice following chronic alcohol exposure by inhalation (Katsura et al. 2005). However, in the model of chronic intermittent alcohol exposure, the protein levels of the L-type $Ca_V1.2$ (α_{1C}) subunit were decreased in the central nucleus of the amygdala (Varodayan et al. 2017b). The dihydropyridine binding sites, which represent L-type $Ca_V1.x$ channels, were increased in ethanol-dependent brains (Dolin et al. 1987). Accordingly, chronic alcohol exposure increased total Ca_V currents including L-type $Ca_V1.x$ in hippocampal neurons in ethanol-tolerant long-sleep mice compared to short-sleep mice; this effect was not associated with changes in the biophysical properties of the channels, suggesting an increase in the number of functional L-type $Ca_V1.x$ channels (Huang and McArdle 1993). L-type $Ca_V1.x$ channels are also implicated in alcohol-mediated neurodegeneration, as inhibition of these channels attenuated cytotoxicity related to chronic alcohol exposure of neocortical cell cultures (Ruhe and Littleton 1994).

Finally, the protein levels of N-type $Ca_V2.2$ (α_{1B}) channels were unchanged in cortical neurons following chronic alcohol administration (Katsura et al. 2005), whereas McMahon et al. (2000) reported an increase in the number of N-type $Ca_V2.2$ channels in the frontal cortex and hippocampus in AWS-prone mice following chronic alcohol administration. Thus, the increase in N-type $Ca_V2.2$ channel expression may be specific to certain brain structures, and this increase may be related to the genetic predisposition of AWS-prone mice to these seizures. Importantly, mice that lack functional N-type $Ca_V2.2$ channels have reduced alcohol consumption (Newton et al. 2004). Similarly, mice treated with blockers and/or agonists of L-type $Ca_V1.x$ channels have reduced alcohol consumption (Rezvani and Janowsky 1990; Rezvani et al. 1991; De Beun et al. 1996a, b). These findings suggest that the anti-alcohol effect may not be related to antagonistic activity at L-type $Ca_V1.x$ channels; alternatively, the anti-alcohol effect may be restricted to specific brain sites. The amygdala appears to be one of the brain sites underlying this behavioral effect, as blocking of L-type $Ca_V1.x$ channels in the central nucleus of the amygdala reduces alcohol intake in rodents (Varodayan et al. 2017b). Taken together, these findings suggest that both L-type $Ca_V1.x$ channels and N-type $Ca_V2.2$ channels might serve as viable therapeutic targets for treating of alcoholism. The mechanisms underlying changes in L-type $Ca_V1.x$ channels and N-type $Ca_V2.2$ channels are not fully understood (Fig. 2). Nevertheless, chronic alcohol exposure increases the expression of protein kinase C (PKC) isoforms, including PKC delta (PKCδ) and PKC epsilon (PKCε); moreover, chronic alcohol exposure upregulated L-type $Ca_V1.x$ channels and N-type $Ca_V2.2$ channels via PKCδ- and PKCε-dependent mechanism, respectively (Gerstin et al. 1998; McMahon et al. 2000).

Interestingly, in primates, chronic alcohol exposure decreases and increases LVA $Ca_V3.x$ in the thalamus and inferior olive, respectively (Carden et al. 2006; Welsh et al. 2011). In contrast, no changes in the mRNA levels or current density of LVA $Ca_V3.x$ channels were seen in thalamic neurons in a mouse model of chronic alcohol exposure (Graef et al. 2011); however, the steady-state inactivation of LVA $Ca_V3.x$ channels was altered in these neurons during alcohol intoxication suggesting a change in Ca^{2+} currents carried by these channels (Graef et al. 2011).

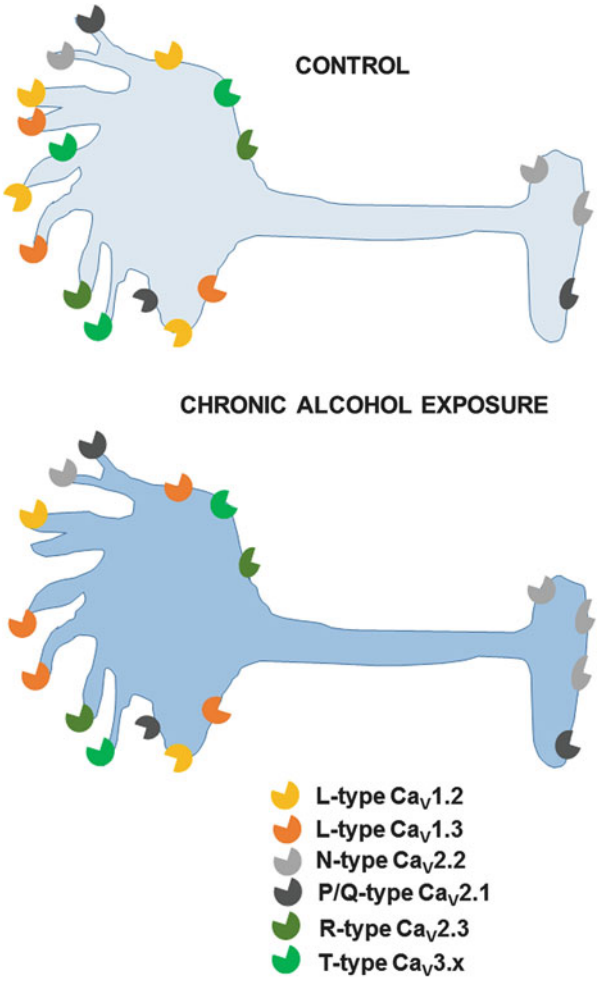

Fig. 2 Chronic alcohol intoxication upregulates Ca_V channels in the brain. During chronic alcohol exposure, a compensatory mechanism to the inhibitory effect of alcohol exposue occurs leading to an upregulation of Ca_V channels including L-type $Ca_V1.3$ (but downregulation of $Ca_V1.2$); moreover, the function and/or expression of these channels is masked by the inhibitory effect of alcohol. LVA Ca_V channels are either upreglated or downregulated in various brain sites

5 Effects of Alcohol Withdrawal on the Expression and Function Ca_V Channels

Alcohol withdrawal triggers increase in the expression of early gene c-fos throughout the CNS at the time at which the seizure susceptibility peaked (Bouchenafa and Littleton 1998). The increased expression of c-fos was prevented by inhibition of L-type $Ca_V1.x$ channels, suggesting an important role of Ca^{2+} influx in the mechanisms underlying AWS susceptibility (Bouchenafa and Littleton 1998). In addition, withdrawal from chronic alcohol exposure induced neuronal hyperexcitability in the hippocampus; this epileptiform activity was mediated, in part, by L-type $Ca_V1.x$ channels (Riplet et al. 1996; Whittington and Little 1991, 1993; Whittington et al. 1992, 1995). Seizures are usually the most severe symptoms

associated with alcohol withdrawal syndrome. Typically, these AWSs are generalized tonic-clonic seizures, which are initiated in the brainstem. In our model of acoustically evoked AWSs, neurons in the IC play a critical role in initiating AWSs, whereas the cortex, hippocampus, and amygdala play a role in propagating these seizures (Faingold et al. 1998; Takao et al. 2006; Faingold 2008; Newton and N'Gouemo 2017). In this model, K^+ depolarization–induced Ca^{2+} transients were increased in inferior colliculus neurons when the susceptibility to AWS peaks (our unpublished data). The influx of Ca^{2+} into neurons plays an important role in the neuronal hyperexcitability that underlies seizures, as $[Ca^{2+}]_i$ rises – and extracellular $[Ca^{2+}]$ decreases – during epileptiform activity (Heinemann et al. 1977; Albowitz et al. 1997; Delorenzo et al. 2005). Thus, inhibition of Ca^{2+} influx into neurons is a promising therapeutic approach for various types of seizures, including AWSs. Interestingly, pharmacologically blocking L-type $Ca_V1.x$ channels suppressed acoustically evoked AWSs (Little et al. 1986). These findings suggest that altered L-type $Ca_V1.x$ channels – at least in the IC – play a key role in initiating these seizures. Consistent with this notion, currents through HVA Ca_V channels are increased before the onset of AWS susceptibility and when the prevalence of AWSs peaks, but they returned to control levels after AWS susceptibility has returned to baseline (N'Gouemo 2015; N'Gouemo and Morad 2003). Thus, the increase in HVA Ca_V currents measured in IC neurons *prior* to the onset of AWS susceptibility cannot be a consequence of seizure activity. Interestingly, alcohol withdrawal increased HVA Ca_V currents in dentate granule neurons in AWS-prone mice but not in AWS-resistant mice (Perez-Velazquez et al. 1994), suggesting that genetic differences in the genes encoding HVA Ca_V channels may contribute to differences in AWS susceptibility and the expression of HVA Ca_V channels.

Alcohol withdrawal-induced upregulation of L-type $Ca_V1.x$ channels in the brain was also reported in a mouse model (Brennan et al. 1990; Guppy et al. 1995; Watson and Little 1999). In our rat model of acoustically evoked AWSs, the increased Ca^{2+} current density in IC neurons mediated by L-type $Ca_V1.x$ channels and P-type $Ca_V2.1$ channels occurs during peak AWS susceptibility (N'Gouemo 2015; N'Gouemo and Morad 2003). These findings suggest a possible causal relationship between the upregulation of L-type $Ca_V1.x$ channels and P-type $Ca_V2.1$ channels in IC neurons and the occurrence of AWSs. L-type $Ca_V1.x$ channels and P-type $Ca_V2.1$ channels play important roles in synaptic plasticity and glutamate release, respectively (Thiagarajan et al. 2005; Ermolyuk et al. 2013). Thus, an increase in currents through L-type $Ca_V1.x$ channels and/or P-type $Ca_V2.1$ channels in IC neurons is likely to increase both firing and transmitter release, leading to increased AWS susceptibility. Consistent with this notion, blocking L-type $Ca_V1.x$ channels in the IC suppressed AWS susceptibility, whereas inhibiting P-type $Ca_V2.1$ channels only reduced AWS severity (N'Gouemo 2015). Moreover, the protein levels of L-type $Ca_V1.3$ (α_{1D}) channels – but not L-type $Ca_V1.2$ (α_{1C}) channels or P/Q-type $Ca_V2.1$ (α_{1A}) channels – are upregulated in IC neurons when AWS susceptibility peaks (Fig. 3), but not *prior* to the onset of AWS susceptibility (N'Gouemo et al. 2015; Newton et al. 2018). However, it is important to note that the lack of change in protein levels of P/Q-type $Ca_V2.1$ (α_{1A}) channels reflects all P/Q-type channel phenotypes and may therefore masks any increase in the selective expression of P-type $Ca_V2.1$ channels occurring in some selective neuronal subtypes.

Interestingly, although mRNA expression of *CACNA1D* and *CACNA1A* (which encode the L-type α_{1D} and P/Q-type α_{1D} subunits, respectively) is increased in IC neurons prior to the onset of AWS susceptibility, their corresponding total protein levels are unchanged in these neurons (N'Gouemo et al. 2015; Newton et al. 2018). Thus, changes in cell surface expression and/or phosphorylation of these HVA Ca_V channels may account for the increased current density in IC neurons *prior* to the onset of AWS susceptibility. In support of this notion, the activity and expression of protein kinase A are increased in IC neurons *prior* to the onset of AWS susceptibility (Akinfiresoye et al. 2016). Under normal conditions, phosphorylation by protein kinase A enhances L-type $Ca_V1.x$ and P-type $Ca_V2.1$ currents (Fournier et al. 1993; Mogul et al. 1993; Davare and Hell 2003), while activation of PKC inhibits the activity of N-type $Ca_V2.2$ channels, but increases other types of Ca_V currents (Diversé-Pierluissi and Dunlap 1993; Rane and Dunlap 1986; Rane et al. 1989). Interestingly, alcohol acts on L-type $Ca_V1.x$ channels by inhibiting calmodulin-dependent activity of the channel (Canda et al. 1995). Thus, increase in L-type $Ca_V1.x$ currents *prior* to the onset of AWS susceptibility may be due to phosphorylation of the channels. Similarly, downregulation of N-type $Ca_V2.2$ channels seen in IC neurons at the time at which AWS susceptibility peaks may be due to enhanced PKC activity.

On the other hand, the protein levels of N-type $Ca_V2.2$ (α1B) subunit are decreased in IC neurons when AWS susceptibility peaks (N'Gouemo et al. 2006) (Fig. 3). Interestingly, activation of PKC inhibits the activity of N-type $Ca_V2.2$ channels, but increases other types of Ca_V currents (Diversé-Pierluissi and Dunlap 1993; Rane and Dunlap 1986; Rane et al. 1989), suggesting increased PKC activity in the IC following alcohol withdrawal at the time at which the susceptibility to AWS peaked. The downregulation of N-type $Ca_V2.2$ channels may contribute to AWS susceptibility by reducing Ca^{2+}-dependent inhibitory mechanisms, as Ca^{2+} influx contributes to the activation of Ca^{2+}-activated K^+ current, which initiates repolarization and underlies the afterhyperpolarization, an intrinsic neuronal inhibitory mechanism (Faber and Sah 2003; Loane et al. 2007; Berkefeld et al. 2010; N'Gouemo and Morad 2014). Interestingly, some Ca^{2+} channel types have been shown to provide the necessary Ca^{2+} influx required to activate small-conductance, and/or large-conductance, Ca^{2+}-activated K^+ channels in the brain (Faber and Sah 2003; Berkefeld et al. 2010). Thus, there appear to be significant differences in coupling between Ca^{2+} channels and Ca^{2+}-activated K^+ channels, suggesting a functional role for the Ca^{2+} channels in driving the activity of Ca^{2+} microdomains.

In primates, alcohol withdrawal decreases LVA $Ca_V3.x$ currents in inferior olive neurons (Welsh et al. 2011). In a mouse model of alcohol withdrawal, thalamic neurons have increased mRNA levels of the genes encoding the LVA $Ca_V3.2$ and $Ca_V3.3$ channel subtypes, but not $Ca_V3.1$ channel subtype (Graef et al. 2011). Despite these changes in mRNA levels and in the steady-state inactivation of LVA $Ca_V3.1x$ channels, alcohol withdrawal does not cause a change in LVA $Ca_V3.1x$ currents in thalamic neurons (Graef et al. 2011). However, ethosuximide, a potent blocker of LVA $Ca_V3.x$ channels commonly used to treat absence seizures, suppresses susceptibility to AWSs in a mouse model (Riegle et al. 2015), suggesting these channels may have therapeutic applications beyond the treatment of absence seizures.

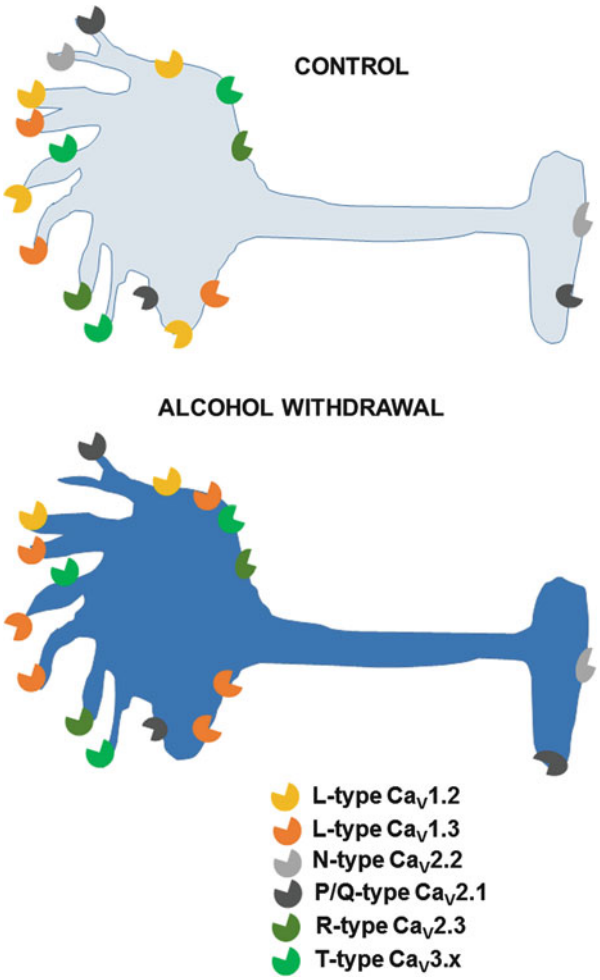

Fig. 3 Alchol withdrawal upregulates Ca_V channels. Alcohol withdrawal following chronic alcohol exposure unmasks the upregulation of Ca_V channels mainly L-type CaV1.3 channels and downregulation of L-type CaV1.2 and N-type $Ca_V2.2$ channels. LVA Ca_V channels are either upreglated or downregulated in various brain sites

6 Conclusion

In the CNS, Ca_V channels play an important role in regulating neuronal excitability, and changes in their activity and/or expression contribute to a wide variety of pathological conditions, including seizures. In keeping with their central role in CNS excitability, Ca_V channels are also an important target for alcohol, and both acute and chronic alcohol exposure, as well as alcohol withdrawal, can alter the function of Ca_V channels, giving rise to an array of symptoms and disorders, including alcohol abuse, alcoholism, and AWSs. Paradoxically, there is a positive relationship between increased Ca_V channel function/expression and increased susceptibility to AWSs, yet downregulating Ca_V channels can also cause seizures, as

some Ca_V channels are functionally coupled to K^+ channels and/or chloride channels. From this review, it becomes clear that HVA $Ca_V1.x$ (i.e., L-type) channels and HVA $Ca_V2.2$ (i.e., N-type) channels are promising targets for treating alcohol abuse and alcoholism; in contrast, L-type $Ca_V1.3$ – and to some extent LVA $Ca_V3.x$ (i.e., T-type) – channels are promising targets for treating AWSs. Moreover, the alcohol-related changes in the function and/or expression of various Ca_V channels vary among brain structures, suggesting the need for targeted therapeutic approaches, reflecting the notion that localized changes in specific Ca_V channels induce distinct sets of symptoms associated with alcoholism and the alcohol withdrawal syndrome.

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Voltage-Sensitive Potassium Channels of the BK Type and Their Coding Genes Are Alcohol Targets in Neurons

Alex M. Dopico, Anna N. Bukiya, and Jill C. Bettinger

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A.M. Dopico (✉) · A.N. Bukiya
Department of Pharmacology, College of Medicine, The University of Tennessee Health Science Center, Memphis, TN, USA
e-mail: adopico@uthsc.edu

J.C. Bettinger
Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA, USA

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_78

Abstract

Among all members of the voltage-gated, TM6 ion channel superfamily, the proteins that constitute calcium- and voltage-gated potassium channels of large conductance (BK) and their coding genes are unique for their involvement in ethanol-induced disruption of normal physiology and behavior. Moreover, in vitro studies document that BK activity is modified by ethanol with an EC_{50}~23 mM, which is near blood alcohol levels considered legal intoxication in most states of the USA (0.08 g/dL = 17.4 mM). Following a succinct introduction to our current understanding of BK structure and function in central neurons, with a focus on neural circuits that contribute to the neurobiology of alcohol use disorders (AUD), we review the modifications in organ physiology by alcohol exposure via BK and the different molecular elements that determine the ethanol response of BK in alcohol-naïve systems, including the role of an ethanol-recognizing site in the BK-forming slo1 protein, modulation of accessory BK subunits, and their coding genes. The participation of these and additional elements in determining the response of a system or an organism to protracted ethanol exposure is consequently analyzed, with insights obtained from invertebrate and vertebrate models. Particular emphasis is put on the role of BK and coding genes in different forms of tolerance to alcohol exposure. We finally discuss genetic results on BK obtained in invertebrate organisms and rodents in light of possible extrapolation to human AUD.

Keywords

Alcohol · *KCNMB* genes · MaxiK channel · Neuron · *Slo1* and orthologs

Abbreviations

ACA	Acetaldehyde
AFT	Acute functional tolerance
AHP	Afterhyperpolarization
AMPA	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
AP	Action potential
AUD	Alcohol use disorders
BK	Voltage- and calcium-gated potassium channel of large conductance
Ca_V	Voltage-gated calcium channel
CIE	Chronic intermittent ethanol
cPC	Cerebellar Purkinje cell
CTD	Cytosolic tail domain
DA	Dopamine
EC	Extracellular
EC_{50}	Ligand concentration at which 50% of the ligand's maximal effect is reached
fAHP	Fast afterhyperpolarization
GABA	4-Aminobutanoic acid

GLUT	Glutamate
HIC	Handling-induced convulsions
I	Macroscopic current
i/IC	Intracellular
Ibtx	Iberiotoxin
KO	Knockout
K_V	Voltage-gated potassium channel
LORR	Loss of righting reflex
MSN	Medium spiny neuron
NMDA	*N*-methyl-D-aspartate
PGD	Pore-gate domain
PKA	Protein kinase A
Po	Open probability
POPE	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
RCK	Regulatory of conductance for potassium
SCN	Suprachiasmatic neurons
SN	Substantia nigra
TM	Transmembrane
VSD	Voltage-sensor domain
VTA	Ventro-tegmental area

1 Introduction

"MaxiK" channels or "BK"[1] are ionotropic, transmembrane receptors defined by the unique coupling of large unitary conductance and selectivity for K^+ over other monovalents to dual activation by increased intracellular Ca^{2+} ($Ca^{2+}{}_i$) and more positive transmembrane voltage. This chapter is focused on BK in neurons and neuroendocrine cells within the brain. Thus, we will not discuss the physiological role and eventual modulation by ethanol of BK in the spinal cord, glia, or cerebral artery tissues (reviewed by Contet et al. 2016; Krishnamoorthy-Natarajan and Koide 2016). Because invertebrate species are often used as model organisms for studies on both BK channels and alcohol, we have included information on the effect of alcohol and related sedatives on invertebrate nervous systems. After a glance at BK molecular assembly and physiological roles in neuronal compartments (Sect. 2), the action of ethanol on BK in alcohol-naïve systems will be

[1] As previously discussed (Dopico et al. 2014), "BK channel" should properly be used to denominate not only $Ca^{2+}{}_i$-activated K^+ channels of large conductance, which are products of the Slo1 gene (slo1 channels) and orthologs, but also the products of Slo2 and Slo3 genes, which render K^+ channels gated by ions other than Ca^{2+}. Thus, slo1 channels should be labeled $BK_{V,Ca^{2+}}$. For consistency with most of the literature and brevity, we will simply use the term "BK" to design a protein complex (with or without regulatory subunits) where the channel-forming subunits are slo1 proteins.

presented[2] (Sect. 3). This information is critical to understand the complexity of mechanisms at the genetic, epigenetic, posttranslational, and/or signaling levels that involve BK subunits or their coding genes in the neurobiological bases of alcohol use disorders (AUD), which are usually associated with repeated and/or protracted exposure of the brain to ethanol (Sect. 4). Due to space limitations, on several points, we refer the reader to previous, expanded reviews where citations to the original articles can be found.

2 BK in Brain Neurons

2.1 Basic Channel Structure, Coding Genes and Brain Expression

BK-forming alpha subunit is the product of a single gene (*KCNMA1* or *Slo1* in mammals, including humans). However, Slo1 pre-mRNA is subject to significant alternative splicing, editing, and further regulation by miRNA (reviewed in Shipston and Tian 2016). The resulting mRNA and protein isoforms vary in brain regional distribution, as well as neuronal trafficking and, thus, organelle distribution. These processes, followed by posttranslational modification of slo1 itself (Kyle and Braun 2014; Shipston and Tian 2016) and its associated regulatory subunits (Lu et al. 2006; Li and Yan 2016), determine the ionic current phenotype, *including its alcohol pharmacology* (see Sects. 3.3 and 4.2).

The slo1 channel signature phenotype stated in the introduction recognizes the structural basis of a modular protein where a pore-gate domain (PGD), a voltage-sensor domain (VSD), and an ion (Ca^{2+} and Mg^{2+})-sensing domain have long been acknowledged. The former two (S0-S6, in which S1-S6 is highly conserved to the core of TM6 K_V channels) and the latter have transmembrane (TMs) and cytosolic locations, respectively (Wang and Sigworth 2009; Yuan et al. 2010; Wu et al. 2010). The so-called cytosolic tail domain (CTD) includes two regulatory of conductance for K^+ structures (RCK1 and 2) where electrophysiological data have mapped two high-affinity, Ca^{2+}-recognition sites (Xia et al. 2002): the "RCK1-Ca^{2+} site" and the "Ca^{2+}-bowl." Thus, in the slo1 homotetramer, Ca^{2+} cooperative binding to both sites expands the octameric (two RCKs per unit) gating ring, which is coupled to the VSD via a short "linker" (Fig. 1). Recent cryo-EM data of a full-length slo1 channel from *Aplysia californica* confirm the location of the bound divalents and reveal both direct interaction of RCK1 with VSD and the stiff nature of the linker, asserting elastic properties to the gating ring itself (Tao et al. 2017).

Differential distribution of BK subunits and slo1 isoforms allows for distinct control of excitability among brain regions and neuronal compartments. For example, a mouse brain slo1 variant containing a terminal insert ("Strex") in the CTD whose exon is regulated by stress hormones is characterized by fast activation, slow deactivation, and increased open probability (Po) at negative potentials when

[2]Unless otherwise stated, statements in this chapter refer to findings obtained in rat or mouse brain and neurons.

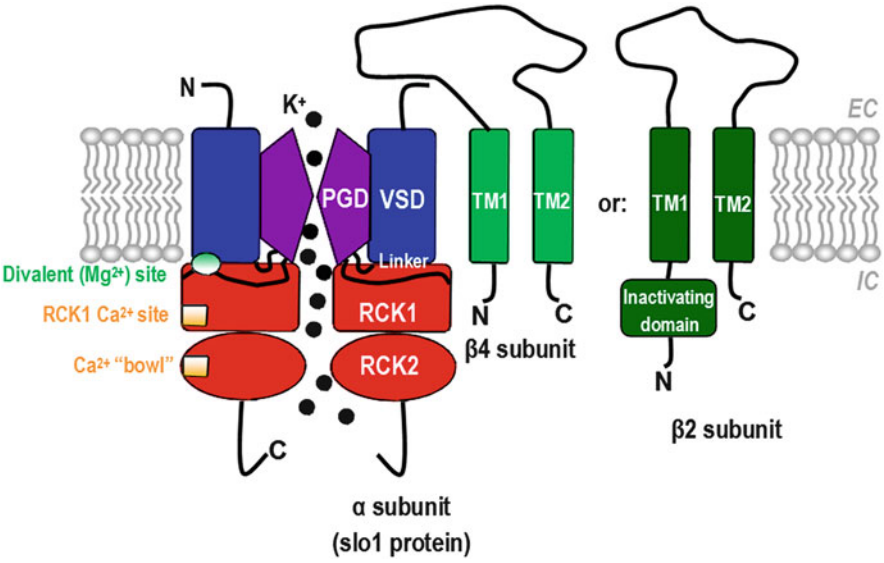

Fig. 1 Cartoon depicting a cross section through two BK α subunits (slo1 proteins) in the presence of BK β4 or β2 regulatory subunits. In most mammalian tissues, BK channels are heteromers of α and β subunits, yet slo1 homotetramers render fully functional BK channels. In central neurons, β4 are widespread and abundant, while β2 and β3 show lesser expression, and β1 is found in selected central neurons. The large EC loop of β4 renders most neuronal BK channels resistant to docking and block by the peptides charybdotoxin and iberiotoxin, while the inactivating domain of β2 emboldens some brain BK channels with distinct contribution to excitability (see main text). BK "γ" subunits (i.e., LRRC proteins) are not depicted as their functional presence in adult brain BK channels remains undetermined. The modular nature of the slo1 protein is underscored: the voltage-sensor domain (VSD, in blue) and the central pore-gate domain (PGD, in purple) are largely embedded in the lipid bilayer with an N-end of extracellular location. The large C-end lies in the intracellular compartment and conforms the cytosolic tail domain (CTD). Each CTD contains two regulatory of conductance for K^+ (RCK) domains (in red). RCK1 includes binding sites for physiological levels of Ca^{2+}_i (in shaded orange) and Mg^{2+}_i (in shaded green), while RCK2 includes another site for recognition of physiological Ca^{2+}_i ("calcium bowl," also in shaded orange). The resulting octameric RCK structure conforms the gating ring of the homotetrameric channel. VSD and gating ring are connected to the PGD by linkers (black line) and through domain-domain interface contacts. With modifications, from Magleby (2017); in turn, based on data from Tao et al. (2017)

compared to the insert-less, "zero" variant (Xie and McCobb 1998). Similar results were obtained when slo1 isoforms were studied after heterologous expression (Poulsen et al. 2011). This study revealed the presence of a brain-specific "X1" variant having three inserts (in the S1-S2 EC loop, between RCK1 and RCK2, and distal to the Ca^{2+} bowl) that displayed faster activation and reduced voltage sensitivity and current than the zero variant. Remarkably, Strex is reduced after birth, probably to allow non-Strex variants to exert a more efficient brake on hyperexcitability (MacDonald et al. 2006; Contet et al. 2016). Strex isoforms remain detectable in CA3 pyramidal neurons, dentate gyrus granule cells, and cerebellar Purkinje cells (cPCs) (Contet et al. 2016). All these brain neurons

experience significant development and maturation after birth. It may be suggested that the Strex phenotype enables an increased degree of excitability that is critical for neurocircuitry formation.

In most mammalian tissues, BKs include small, two TMs, regulatory subunits (β) associated with slo1 proteins (i.e., BK α subunits) (Fig. 1). BK "γ" subunits (i.e., LRRC proteins) have also been identified. Both β and γ subunits drastically affect the BK current phenotype (Lu et al. 2006; Li and Yan 2016), yet the functional significance of γ subunits in brain neurons remains to be established. Four β types have been identified (encoded by *KCNMB1–4*). Beta4 and β2 distribution in mammalian brain neurons is widespread, yet β4 reaches significantly higher levels than β2. Beta2, however, includes an IC domain that uniquely confers fast inactivation to the BK current. Beta1 and β3 (variants 3b–d) are expressed at very low levels in most brain areas, yet β1 shows significant expression in cerebellar Purkinje cell (cPCs), many brainstem nuclei, and several hypothalamic regions (Contet et al. 2016; Li and Yan 2016). The subunits confer different pharmacological properties to the BK channel; β4 provides BK resistance to the selective peptide blockers charybdotoxin and iberiotoxin (Ibtx) (Lu et al. 2006), while β1 blunts ethanol-induced potentiation of BK currents and enables ethanol-induced reduction of BK activity at physiological low μM $Ca^{2+}{}_i$ (Dopico et al. 2014). Remarkably, differential co-expression of BK β1 vs. β4 subunits is the primary determinant of the differential alcohol response of BKs across different compartments of the same neuron, e.g., soma vs. nerve endings of supraoptic magnocellular neurons; soma vs. dendrites of nucleus accumbens (NAc) medium spiny neurons (MSN) (reviewed in Dopico et al. 2014, 2016; Contet et al. 2016).

Regarding BK "γ" subunits (1, 2, 3, and 4, encoded by *LRRC26, LRRC52, LRRC55*, and *LRRC38*, respectively), γ1 is abundant in fetal brain, while γ3 and 4 are expressed in adult brain (reviewed by Li and Yan 2016). Data from recombinant BK expressed in *Xenopus laevis* oocytes show that a slo1 + β2 + γ1 complex renders a novel current phenotype, which depends on the stoichiometry of β2 (Gonzalez-Perez et al. 2015). Whether this BK plays a physiological role in fetal brain, where γ1 is abundant, remains to be determined. Likewise, whether another ternary heterotetramer exists and plays a functional role in brain neurons remains unknown.

Finally, several *Drosophila* neural tissues, including photoreceptors, optic lobe, pars intercerebralis neurons, and the surrounding cortex, express Slo-binding proteins (Slobs), which are an integral part of BK complexes and regulate ion current phenotype (Jaramillo et al. 2004).

2.2 General Function in Central Neurons and Neurosecretory Elements

BKs in central neurons play key roles, at both pre- and postsynaptic membranes, in the excitability of somata, axon terminals, and dendrites. Disregarding the particular cell membrane, BK's low affinity for Ca^{2+}, resulting in Ca^{2+} in μM levels to gate the channel at the physiological negative potentials that prevail in neurons, requires

BK to be located nearby sources of Ca^{2+} influx, including Ca_V (most types), NMDA and AMPA channels, and/or intracellular stores to effectively participate in neuronal firing, neurotransmitter release, or neurotoxicity. Such BK clustering near Ca^{2+}-signaling elements, usually in nanodomains (Fakler and Adelman 2008; Vandael et al. 2010; Contet et al. 2016), also allows BK to effectively control excitability without needing a widespread increase in $Ca^{2+}{}_i$, which could lead to neurotoxicity. However, local clustering of Ca^{2+} influx conductances and BK may facilitate depolarization and/or neurotoxicity. Thus, Ca^{2+} influx via glutamate (GLUT) receptors in mouse cortical layer 5 pyramidal neurons evokes increased activity of nearby BK within seconds of hypoxia-induced depolarization (Revah et al. 2016). In contrast, neuroprotective effects of BK against GLUT neurotoxicity were reported in slices from the hippocampus (Rundén-Pran et al. 2002) and the cortex-striatum (Katsuki et al. 2005). Consistent with this protective role, BK activation by cytokine IL-10 reduces hypoxia-induced increase in excitability in hippocampal CA1 pyramidal neurons (Levin et al. 2016). The role of BK modulation by oxidative stress in neurotoxicity has been extensively reviewed elsewhere (Hermann et al. 2015).

BK contributes to action potential (AP) single spike shape and firing patterning, yet the final effect on neuronal firing may be an actual *decrease or increase* based on the specific BK subunits present, BK coupling to other conductances (e.g., I_m, I_h; Ly et al. 2011), and/or distinct signaling within a given neuronal compartment or brain region. "Neuronal," i.e., *β4-containing*, BK broadens APs and increases the fast afterhyperpolarization (fAHP), as widely reported across most brain regions (Contet et al. 2016). Genetic ablation of *KCNMB4* in rat hippocampal dentate gyrus granule neurons, however, leads to AP sharpening, increased fAHP amplitude, and enhanced spike frequency, underscoring a pro-excitatory role for BK-mediated fAHP (in the absence of β4). Work with engineered proteins has revealed that β4 decouples slo1 from RyR leading to reduced excitability (Wang et al. 2016). In general, fast-inactivating BK activity in high-frequency neurons contributes to fAHP and decreases firing rate, whereas BK exerts opposite effects in neurons with low spontaneous firing. An enhanced fAHP may also speed up Na^+ channel recovery from inactivation and limit the activation of slow K_V outward rectifiers, as found in CA1 pyramidal neurons, leading to faster firing (Gu et al. 2007; Contet et al. 2016).

In substantia nigra (SN) dopamine (DA) neurons, inhibition of BK expectedly leads to wider APs due to slow repolarization. However, fAHP does not decrease but actually increases, this effect being attributed to recruitment of additional current via nearby K_V2 channels (Kimm et al. 2015). The relative contribution of Kv channels vs. BK to repolarization often differs between different neuronal populations within a given brain area. Thus, in mouse cortex, BK plays a significant role in shaping both somatic and axonal APs of somatostatin-expressing interneurons. In turn, K_V1 channels seem to only play a major role in shaping axonal APs of GABA-releasing basket interneurons (Casale et al. 2015).

At chemical presynaptic endings and terminals of neurosecretory cells, BK usually decreases neurotransmitter (particularly, GLUT; Martire et al. 2010) or

hormone release (e.g., vasopressin, oxytocin, gonadotrophin) by opposing the depolarization evoked by incoming APs. Likewise, Ibtx has been reported to increase the probability of GABA and glycine release in central amygdala and spinal cord neurons, respectively. In a few species (mostly nonmammalian), however, BK mediates the opposite effect on neurotransmitter release (reviewed in Contet et al. 2016).

In apical dendrites of hippocampal CA1 pyramidal neurons, BK activation limits repetitive firing of Ca^{2+} spikes (Golding et al. 1999). BK-mediated reduced propagation of dendritic Ca^{2+} spikes was also observed in cPC (Rancz and Häusser 2006). The role of BK in backpropagating APs seems to depend on species and/or brain regions. Thus, in the leech anterior pagoda neuron, BK inhibition leads to increased amplitude of backpropagating Na^{+} spikes (Wessel et al. 1999). In contrast, dendritic BK regulates neither amplitude nor duration of backpropagating APs in cortical layer 5 pyramidal neurons of the rat, while its somata counterpart does contribute to AP repolarization and to mediating fAHP (Bock and Stuart 2016).

In addition to the neuronal plasma membrane, BK have been mapped to internal organelles, including the inner mitochondrial membrane (Douglas et al. 2006) and the nuclear envelope, with nuclear BK-regulating gene expression (Fedorenko et al. 2010; see Balderas et al. 2015; Li and Gao 2016 for reviews). A proteonomic analysis of mouse brain combining mass spec, histochemistry, and confocal microscopy revealed several hundred unique proteins in the BK interactome, including pre- and postsynaptic, mitochondrial, and nuclear interacting partners for slo1 (Singh et al. 2016).

Consistent with their widespread participation in regulating excitability across all neuronal compartments, (a) *KCNMA1*$^{-/-}$ mice suffer a variety of severe neurological dysfunctions (Zemen et al. 2015), and (b) BK-mediated currents become prominent at developmental times that define maturation of a given CNS structure, e.g., in the early postnatal period for pyramidal neurons in the neocortex or SN DA neurons (reviewed in Contet et al. 2016). However, BK expression and impact on excitability across the different neuronal compartments shows regional variability. For instance, slo1 immunoreactivity of hippocampal pyramidal cells is higher in presynaptic membranes and in dendritic areas that oppose postsynaptic contacts, whereas cPCs show higher slo1 channel levels in dendritic extrasynaptic membranes away from postsynaptic contacts (reviewed in Contet et al. 2016).

BK's sensitivity to $Ca^{2+}{}_{i}$ is likely a determining factor for the channel's involvement in neuronal plasticity and neuroprotection/neurotoxicity. Indeed, BK current in pyramidal neurons of the prefrontal cortex displays drastic changes during adolescence, a period in which such neurons are characterized by enhanced plasticity (Contet et al. 2016). In turn, BK activity is usually associated with neuroprotection. For instance, in mouse cortical neurons, prostaglandin E2 receptor signaling evokes NMDA-mediated currents that lead to BK activation and, thus, reduced dendritic beading (Hayashi et al. 2016). However, BK activation through NLRP1, a member of the inflammasome complex, by chronic levels of glucocorticoids has been linked to degeneration of hippocampal neurons (Zhang et al. 2017).

After describing BK general roles in the different neuronal compartments, we will discuss the importance of this channel in distinct brain regions, with a focus on brain neurons known to be affected by ethanol exposure and/or involved in the neurocircuitry of AUD. BK roles in CNS disease, including epilepsy, motor disorders, memory and cognitive disorders, and cerebral ischemia, have been reviewed elsewhere (Contet et al. 2016).

2.3 Role in Neurocircuits That Contribute to the Biology of Alcohol Use Disorders

Slo1 mRNA and protein levels are detected in almost all brain regions, with particularly high levels in the olfactory bulb, cortex, basal ganglia, thalamus, hypothalamus, cerebellum, and vestibular nuclei (Contet et al. 2016). As indicated above, the "neuronal" β4 subunit also shows high and widespread distribution in the brain. It is noteworthy that in hippocampus pyramidal cells, the high expression of this subunit does not translate into prominent β4-containing currents, and the BK "neuronal" subunit is thought to blunt forward trafficking of slo1 in these cells (Shruti et al. 2012). Remarkably, many brain regions that are very sensitive to intoxicating levels of ethanol and/or participate in the neurobiology of AUD, such as the cerebellum, neocortex, hippocampus and lateral amygdala, include β2-containing BK, which are endowed with fast inactivation (Contet et al. 2016). This fast inactivation, however, does not seem to play a major role in the sensitivity of recombinant β2-containing BKs to acute exposure to intoxicating levels of ethanol (Kuntamallappanavar and Dopico 2016).

DA neurons of the ventro-tegmental area (VTA) are at the center of the "reward system of the brain." In cultured DA neurons from both VTA and SN, selective block of BK with paxilline results in AP widening, reduced amplitude, and reduced fAHP, exemplifying the "classic" roles for BK in AP sharpening. In a more complex system, however, DA projections from VTA inhibit AP firing via dopamine receptor activation in a population (25%) of NAc MSN (Ji and Martin 2014). This action is prevented by paxilline; thus, BK activity plays a critical role in DA receptor-mediated inhibition of some MSNs (Ji and Martin 2014). Opposite effects of BK block on neuronal firing rate may arise from differential BK subunit distribution in different neuronal populations (Brenner et al. 2000; Salzmann et al. 2010), contribution of players additional to BK (including other ion channels) to spike frequency regulation (Sengupta et al. 2010), and from the location of BK along the pathways that controls spike firing (e.g., upstream vs. downstream of DA receptor signaling). Thus, an upstream location of BK would result in paxilline-induced loss of DA receptor-meditated inhibition of MSN firing and, therefore, would lead to an increased MSN activity.

Paxilline actions on CA1 pyramidal neuron firing are similar to those described above for the VTA (Springer et al. 2015). In the lateral amygdala, a key component of the reward system-stress-fear "axis," a decrease in BK current likewise leads to spike widening and increased excitability (Faber and Sah 2003), both phenomena

being significantly higher in fear-conditioned mice vs. controls (Sun et al. 2015). BK-mediated fAHP in these neurons is also reduced by stress-evoked anxiety (Guo et al. 2012). Low-frequency firing neurons from the external globus pallidus, where BK mediates an alcohol-induced decrease in firing rate, show that pharmacological block of BK leads to a mild increase in firing rate (Abrahao et al. 2017). In the cerebellar cortex, another region that is highly sensitive to intoxicating ethanol, pharmacologic block of BK leads to reduced AHP and enhanced cPC firing (Edgerton and Reinhart 2003). BK activity in cPC axons (the only output of the cerebellar cortex) facilitates the inhibitory synaptic response in the deep cerebellar nuclei (Hirono et al. 2015). Also in the cerebellar cortex, BK have been found in Golgi cells (Cheron et al. 2009) and mediate a large component of the depolarization-evoked, non-inactivating K^+ current in stellate cells (Liu et al. 2011). Along the cerebellum, vestibular nuclei participate in vestibule-ocular disorders, including those evoked by drug (e.g., alcohol) exposure. BK critically controls the excitability of medial vestibular nucleus neurons in the brainstem. Decreased BK current with eventual hyperexcitability may be triggered by vestibular damage, and the compensatory increase in eye movements that restore oculomotor function is blunted in slo1 knockout (KO) mice (Nelson et al. 2017).

In thalamocortical relay neurons, BK inhibition prevents spike frequency adaptation; these channels also participate in epileptic activity (Ehling et al. 2013). The role of BK in the neurobiology of seizures and epilepsies has been reviewed elsewhere (N'Gouemo 2011). Growing evidence supports a role for circadian system disruption in AUD. In suprachiasmatic nucleus (SCN) neurons, *inactivating* BK currents increase during the day leading to increased SCN firing. Inactivation is lost after *KCNMB2* ablation, suppressing the diurnal variation in BK current amplitude and SCN firing, both of these defects being rescued by incorporation of β2-mediated inactivation (Whitt et al. 2016).

3 BKs as Targets That Participate in Ethanol Actions on Alcohol-Naïve Systems

3.1 The slo1 Channel Protein and Coding Genes as Ethanol Targets

Numerous reports point at slo1 channel proteins and their coding genes as molecular targets of alcohol in alcohol-naïve preparations during brief exposure episodes. Following a single brief exposure episode, the sedative benzyl alcohol upregulated slo1 gene expression via alteration of histone acetylation in the Slo promoter region of *Drosophila melanogaster* (Wang et al. 2007). In higher organisms, studies of alcohol actions and Slo1 genes and their products have generally focused on ethyl alcohol (ethanol) and how such exposure alters protein function, i.e., BK current. Ethanol-induced activation of BK current was observed in rat neurohypophysial terminals (Dopico et al. 1996; Knott et al. 2002), cell-attached patches from external globus pallidus neurons (Abrahao et al. 2017), as well as in cell-free

membrane patches from numerous cell types, including NAc MSN (Martin et al. 2004). This phenomenon was replicated when recombinant slo1 channels from brain neurons (mouse and human) were expressed in heterologous systems such as *X. laevis* oocytes and HEK cells (Dopico et al. 1998; Feinberg-Zadek et al. 2008). The ethanol-induced activation of neuronal slo1 is concentration-dependent and occurs at physiologically relevant ethanol levels (10–200 mM), with the EC_{50} reported at ~25 mM (Dopico et al. 1998). The EC_{50} of the recombinant mslo1 is close to EC_{50} of native BK in rat neurohypophysial terminals (Dopico et al. 1996). Thus, ethanol modification of slo1 function is conserved in the absence of intracellular signaling and when probed in a mammalian vs. an amphibian cell membrane. Moreover, ethanol-induced activation of neuronal slo1 (hslo1) was detected in an overly simplified system, that is, a planar lipid bilayer formed by only two phospholipid species (Crowley et al. 2003; Yuan et al. 2008), underscoring that ethanol action does not require complex lipid domains associated with the existence of many lipid species in the membrane.

Ethanol action on BK current does not involve modification of N (e.g., number of active channels), current rectification, or unitary slope conductance (Dopico et al. 1996; Jakab et al. 1997; Crowley et al. 2003) but stems from an ethanol-driven minor increase in mean open times (Jakab et al. 1997) and a robust decrease in closed times (or increased frequency of channel openings; Dopico et al. 1996, 1998; Abrahao et al. 2017).

In addition to potentiation of BK current, refractoriness and even inhibition of current by ethanol were reported (see Table I in Dopico et al. 2016). This inconsistency in ethanol modulation of BK function may arise from numerous factors (see below). In cell-free systems, however, ethanol responses of homomeric slo1 were reported to be tuned by a Ca^{2+}-dependent mechanism: ethanol failed to gate slo1 in the absence of activating Ca^{2+}, increased slo1 activity in the presence of physiologically relevant Ca^{2+} concentrations (<10 μM), and decreased slo1 current when Ca^{2+} exceeded 10 μM at the intracellular membrane leaflet (Liu et al. 2008). Ethanol-induced activation of slo1 from mammalian species was sustained in the presence of either of the high-affinity Ca^{2+} sensors (Ca^{2+} bowl and RCK1 domain; see Fig. 1). This conclusion was reached by comparing ethanol action on recombinant channels that contained mutations of either vs. both high-affinity, Ca^{2+}-sensing regions (Liu et al. 2008). A recent study utilizing Horrigan-Aldrich detailed gating analysis demonstrated that ethanol-induced slo1 activation was driven by an increase in the channel's apparent Ca^{2+} affinity in the presence of ethanol. The increase in Ca^{2+} affinity was not accompanied by detectable changes in the channel's intrinsic gating or voltage-dependent parameters. Ethanol did, however, mildly decrease allosteric coupling between Ca^{2+} binding and channel opening (Kuntamallappanavar and Dopico 2016).

With regard to ethanol-induced slo1 inhibition at Ca^{2+} levels exceeding 10 μM, at this Ca^{2+} concentration, channels enter a low Po gating mode that resembles that observed as a result of ligand-driven desensitized states (Liu et al. 2008; Dopico and Lovinger 2009). Ethanol favors the transition of the channel to this low-Po mode, with the RCK1 high-affinity site being necessary to enable ethanol-induced

inhibition at higher Ca^{2+} levels (Liu et al. 2008). Whether these findings on Ca^{2+}-dependent modulation of ethanol effect in mammalian slo1 apply to other species remains unclear; in *Caenorhabditis elegans*, SLO1's Ca^{2+}-sensing domains are not linked to ethanol sensitivity, although they greatly modulate basal channel function (Davis et al. 2015).

In contrast to $Ca^{2+}{}_i$, Mg^{2+}, another intracellular divalent cation that can gate the slo1 channel (albeit via the low-affinity divalent RCK1 binding site; Fig. 1), is not necessary for ethanol to increase mouse slo1 (mslo1) current (Liu et al. 2008). Moreover, recent work on excised patches from hippocampal neuronal cultures shows that ethanol produces BK inhibition at intracellular Mg^{2+} $<$200 μM, with ethanol-induced BK activation being observed at 1 mM Mg^{2+} (Marrero et al. 2015). The latter value is close to the estimated physiological level of intracellular Mg^{2+} in excitable cells (Hille 2001).

Ethanol's ability to augment homomeric slo1 function at physiological divalent cation concentrations in such simple experimental systems as excised membrane patches and artificial lipid bilayers led to the conclusion that the ethanol effect was likely mediated by a molecular target common to all systems: the slo1 protein itself. Based on structural criteria for ethanol-sensing sites that have been put forward from analysis of a variety of proteins (Dwyer and Bradley 2000; Harris et al. 2008), and using computational protein homology modeling of the mslo1 CTD based on crystallographic data from human slo1 (hslo1; Yuan et al. 2010), an alcohol-sensing site was mapped within the mslo1 CTD (Bukiya et al. 2014). This site was characterized as a secluded cavity within the surface of the CTD facing the aqueous intracellular medium. The ethanol-sensing site has estimated dimensions of 10.7 $\times$ 8.6 $\times$ 7.1 Å and is able to accommodate n-alkanols from ethanol up to heptanol. Longer-chain alkanols, such as octanol and nonanol, are unable to fit the ethanol-sensing cavity (Bukiya et al. 2014) and, thus, failed to activate mslo1 channels (Chu and Treistman 1997; Bukiya et al. 2014). Ethanol's fit within the cavity enables hydrogen bond formation with K361. Remarkably, the alcohol-sensing site is adjacent to the RCK1 high-affinity Ca^{2+}-sensing site, with interaction between the two sites (for ethanol and Ca^{2+} binding) being coupled via R514 to the slo1 channel gate. In the absence of Ca^{2+}, this residue points away from the alcohol-docking area (Wu et al. 2010; Bukiya et al. 2014). Thus, R514 is unable to participate in its positive charge-mediated stabilization of ethanol docking. The critical role of R514 in ethanol sensing of mslo1 was confirmed using computational modeling and point mutagenesis (Bukiya et al. 2014). In addition, spatial rearrangement of several other residues within the ethanol-sensing area in Ca^{2+}-free solution was expected to further preclude effective ethanol docking into the sensing area. These data provided a structural explanation for the inability of ethanol to modify BK current in Ca^{2+}-free solution (Liu et al. 2008). An ethanol-sensing region was also mapped in *C. elegans* SLO-1, where T381 mutations blunted SLO-1 ethanol sensitivity (Davis et al. 2014). This site is not identical to that in mslo1, and the discrepancy may be linked to the differential roles of Ca^{2+} sensing in the ethanol response of *C. elegans* (Ca^{2+} is not critical; Davis et al. 2015) vs. mammalian species (Ca^{2+} is necessary, Liu et al. 2008). However, the residue

equivalent to *C. elegans* SLO-1 T381 in mammals (T352) is located near K361. Thus, although the specific residues that interact with ethanol vary, the overall positioning of the alcohol-sensing region within the BK α subunit CTD seems to be evolutionary conserved.

3.2 The slo1 Channel Protein Response to Ethanol: Slo1 Splicing and Posttranslational Modifications of slo1 Proteins

Based on the previous observation that bovine slo1 (bslo1) differed from mslo1 in ethanol sensitivity (Liu et al. 2003), CaM kinase II phosphorylation of T107 in the slo1 S0-S1 linker was shown to override ethanol-induced potentiation of channel activity, even leading to channel inhibition in response to ethanol (Liu et al. 2006). Thus, even a difference in a single amino acid within the slo1 sequence may drastically modulate ethanol sensitivity of slo1. Slo1 isoforms containing T107, however, are largely restricted to the bovine species, whereas slo1 from the vast majority of tissues and species, including humans, contain nonphosphorylatable residues in position 107 or equivalent, this structural feature allowing channel activation by ethanol (Liu et al. 2006).

Ethanol sensitivity of the slo1 channel is also governed by trafficking of slo1 to the site of ethanol action. ERG-28 (endoplasmic reticulum membrane protein) has been recently shown to protect *C. elegans* SLO-1 subunits from proteolytic degradation during their trafficking to presynaptic terminals. As a result, in the absence of ERG-28, SLO-1 channels undergo proteolysis, resulting in markedly reduced expression at presynaptic terminals and diminished alcohol sensitivity of *C. elegans* as measured from a behavioral phenotype (Oh et al. 2017).

3.3 Modifiers of the slo1-Ethanol Interactions: Lipids, BK Accessory Subunits, and Cell Microenvironment

It is widely recognized that membrane lipids play a critical role in tuning BK responses to alcohol. Cholesterol, a major structural lipid in the plasma membrane of eukaryotes, antagonized alcohol-induced BK activation in planar lipid bilayers following reconstitution of recombinant slo1 protein from human brain (Crowley et al. 2003). The presence of cholesterol in the bilayer at physiologically relevant molar fractions (up to 49 mol%) gradually shifted channel open-time distribution histograms to shorter open times while progressively increasing channel closed times, effects that were opposite to those of ethanol on slo1 channels. Thus, cholesterol antagonized ethanol's effect on channel dwelling. At low molar fractions (e.g., $<$20%), however, cholesterol still supported ethanol-induced channel activation (Yuan et al. 2011). The mechanisms behind cholesterol antagonism of ethanol action on BK current remain under investigation and may include indirect (bilayer lipid-mediated) and/or direct (e.g., competition for similar sensing site on BK protein) components. The former would be mediated by cholesterol-driven changes in

membrane physical properties, such as membrane order, lipid packing, membrane dipole potential, and/or thickness (reviewed by Dopico et al. 2012; Gumí-Audenis et al. 2016). A lipid-mediated component in ethanol-cholesterol antagonism on BK function is consistent with the fact that the action of each individual agent on slo1 channels is similarly decreased when probed in one phospholipid species bilayer (POPE; see discussion in Crowley et al. 2003). Remarkably, alcohol-induced BK activation inversely correlates with membrane thickness (Yuan et al. 2007). Thus, it is conceivable that cholesterol antagonism of ethanol-driven increase in BK current may arise from cholesterol's ability to increase membrane thickness (Hung et al. 2007). Changes in membrane physical properties are likely to underlie modulation of the effect of ethanol by bulk membrane phospholipids. It was demonstrated that ethanol-induced potentiation of hslo1 activity in planar lipid bilayers was blunted by type 2 phospholipids but supported by cylindrical ones (Crowley et al. 2005). It was speculated that cylindrical phospholipids could relieve bilayer stress when compared to the inclusion of type 2 lipids, and, thus, bilayer stress could represent a driving force behind lipid-mediated modulation of slo1 ethanol sensitivity (Crowley et al. 2005).

The possibility of direct ethanol sensing by slo1 protein also received experimental support, as enantiomeric cholesterol (*ent*-cholesterol) was unable to modulate ethanol sensitivity of hslo1 to 50 mM ethanol in planar lipid bilayers while changing bilayer properties similarly to natural cholesterol (Yuan et al. 2011). The location of amino acids that enable cholesterol control over the effect of ethanol on slo1 remains unknown.

Among protein modifiers of the ethanol effect on BK current, accessory BK subunits play a central role. Neuron-abundant human β4 subunit co-expressed with the human BK α-subunit splice variant hbr5 in HEK cells did not prevent alcohol-induced BK current activation observed in homomeric hslo1 channels, yet the ethanol activation of β4 subunit-containing channels was generally smaller than of homomeric slo1 (Feinberg-Zadek and Treistman 2007; Velázquez-Marrero et al. 2014). Remarkably, the slo1 characteristic behavior in response to ethanol was also described when human β4 subunit was co-expressed with rat cerebral artery slo1 (cbv1), which has a 99% amino acid sequence identity with mouse brain slo1 (Liu et al. 2008; Kuntamallappanavar and Dopico 2016). In contrast, β2 (with or without inactivation sequence) rendered a BK with an ethanol sensitivity profile similar to that of β1-containing BK, e.g., drastically different from the ethanol sensitivity that is characteristic of slo1 homomers or β4-containing heteromers. β1 themselves are responsible for the inhibition of BK currents observed at physiological voltages and $Ca^{2+}{}_i$, and the resulting alcohol-induced cerebral artery constriction, raising the possibility that BK β1-targeting drugs could be used to counteract alcohol-induced constriction of brain arteries (Kuntamallappanavar and Dopico 2016, 2017). The mechanisms underlying β subunit modulation of BK's ethanol sensitivity may include several non-mutually exclusive possibilities, ranging from the existence of an alcohol-sensing site on BK β subunits and expanding to β subunit modulation of slo1 Ca^{2+} sensitivity and/or protein kinase regulation of slo1 (Feinberg-Zadek and Treistman 2007; Kuntamallappanavar and Dopico 2016).

Protein phosphorylation was shown to play a critical role in tuning BK responses to ethanol. Indeed, the potentiation of BK current in GH3 pituitary tumor cells by ethanol was blocked by PKC inhibitors, but not by a phospholipase C blocker (Jakab et al. 1997). In contrast, phosphatase inhibitors promoted ethanol-induced BK activation (Jakab et al. 1997). Ethanol-mediated enhancement of human slo1 channel activity in HEK cells was completely blocked by a PKA inhibitor (Velázquez-Marrero et al. 2014). Yet, PKA block did not affect the ethanol-induced BK activation in BK heteromers containing β4 subunits (Velázquez-Marrero et al. 2014). Therefore, β subunits may reshape protein kinase modulation of slo1 channels: in the case of β4, these accessory subunits are able to override the modulatory influence of PKA over slo1 ethanol sensitivity.

The effect of ethanol on BK current may also be modulated by alcohol metabolites, such as acetaldehyde (ACA). Ethanol-induced BK activation observed in excised patches from GH3 pituitary tumor cells was progressively suppressed by increasing ACA concentrations at the intracellular side of the membrane leaflet (Handlechner et al. 2013). Whether acetaldehyde presence plays a role in modulating ethanol action on neuronal BK channels remains to be determined.

3.4 Physiological and Behavioral Modifications Consequent to Exposure of BK to Brief Application of Ethanol in Alcohol-Naïve Systems

Consistent with the commonly observed slo1 channel activation by ethanol, this drug is expected to exert an inhibitory effect on neuronal activity. Indeed, behavior patterns of *slo-1* gain-of-function mutants resembled those of *C. elegans* under alcohol intoxication (Davies et al. 2003). Moreover, mutations in *C. elegans slo-1* were found to cause resistance to alcohol intoxication in this model organism (Davies et al. 2003). Accordingly, reduction in functional SLO-1 channels in presynaptic terminals due to accelerated degradation in ERG-28-lacking worms also resulted in an alcohol-resistant phenotype (Oh et al. 2017).

Ethanol-induced reduction in AP firing rate and, thus, decreased neuronal excitability have been reported in several preparations. These include NAc MSN in rat and mouse (Martin et al. 2004, 2008). Ethanol-driven decrease in MSN excitability has been linked to ethanol- perturbation of motor behavior and alcohol preference (reviewed by Treistman and Martin 2009; Bettinger and Davies 2014). Tonically active neurons in mouse external globus pallidus show the critical role of BKs in ethanol-induced reduction of firing rate of low-frequency neurons in this brain region: in the presence of penitrem A, a BK antagonist, the ethanol effect is lost (Abrahao et al. 2017). In supraoptic neurons acutely dissociated from rat, however, BKs were ruled out as major contributors to the ethanol-driven decrease in single evoked spike duration (Widmer et al. 1998). Differential involvement of BK in ethanol effect on excitability may arise from variability in BK subunits (Brenner et al. 2000; Salzmann et al. 2010) and role of ethanol targets other than BK, such as voltage-gated Ca^{2+} and small conductance Ca^{2+}-activated K^+ channels

(Mulholland et al. 2011; reviewed by Harrison et al. 2017). Considering that the final ethanol effect on BK is given by several factors (Dopico et al. 2016; see Fig. 2a), it comes as no surprise that ethanol action on neuronal firing not always result in decreased AP frequency. Indeed, sedation with benzyl alcohol increased neuron excitability within the *D. melanogaster* giant fiber pathway, this effect being accompanied by a substantial decrease in the neuronal refractory period (Ghezzi et al. 2010). Alcohol-driven increase in neuronal firing was dependent on functional slo and could be mimicked by slo gene induction. As a result, flies developed tolerance to repeated alcohol exposure, and slo-mediated increase in neuronal firing rate predisposed animals to seizures. The latter constitutes a characteristic symptom of alcohol withdrawal. Thus, alcohol-driven alteration of BK channel expression/function might provide an explanation for the long-standing counteradaptive theory of "drug tolerance," that is, a homeostatic adaptation(s) that limits the effect of the drug, yet it contributes to dependence upon drug clearance (Cowmeadow et al. 2005; Ghezzi et al. 2010). Alcohol-induced increase in neuronal excitability is not limited to *D. melanogaster*: pharmacological block of presynaptic BKs prevented ethanol-induced enhancement of GABAergic inhibitory transmission in the rat central nucleus of the amygdala (Li et al. 2014).

The complexity of ethanol action at the cellular and organ levels is further increased by the fact that ethyl alcohol is a very simple molecule, a feature that allows this alcohol to "fit" in many putative docking areas (whether within lipid or protein species) and readily cross membranes. Thus, besides modulating BK function, ethanol has the capability to almost simultaneously target several receptors within a single-cell domain. For example, in rat neurohypophysial terminals, ethanol-induced potentiation of BK current was accompanied by ethanol inhibition of Ca_V channels, both ethanol actions contributing to decrease neuropeptide release (Knott et al. 2002). Longer exposure to ethanol, however, triggered compensatory modifications in BK and Ca_V channel density, with consistent reduction in alcohol-induced inhibition of neuropeptide release (Knott et al. 2002).

4 BK and Coding Genes as Mediators of Physiological and Behavioral Alterations Evoked by Protracted and/or Repeated Ethanol Exposure

4.1 Slo1 and Related Genes as Participants of Acute and Rapid Tolerance to Ethanol and Physiological Dependence

In *C. elegans*, SLO-1 is a major mediator of acute behavioral responses to ethanol. Mutations in *slo-1* were identified in a forward genetic screen as they conferred a very strong resistance to the depressing effects of ethanol, consistent with SLO-1 being an important target of ethanol for mediating its behavioral effects (Davies et al. 2003). Like mammals, *C. elegans* develops acute functional (within session) tolerance (AFT) to ethanol within 30 min of an exposure (Davies et al. 2004), and *slo-1* also plays a role in this process. Mutations in *slo-1* diminish but do not

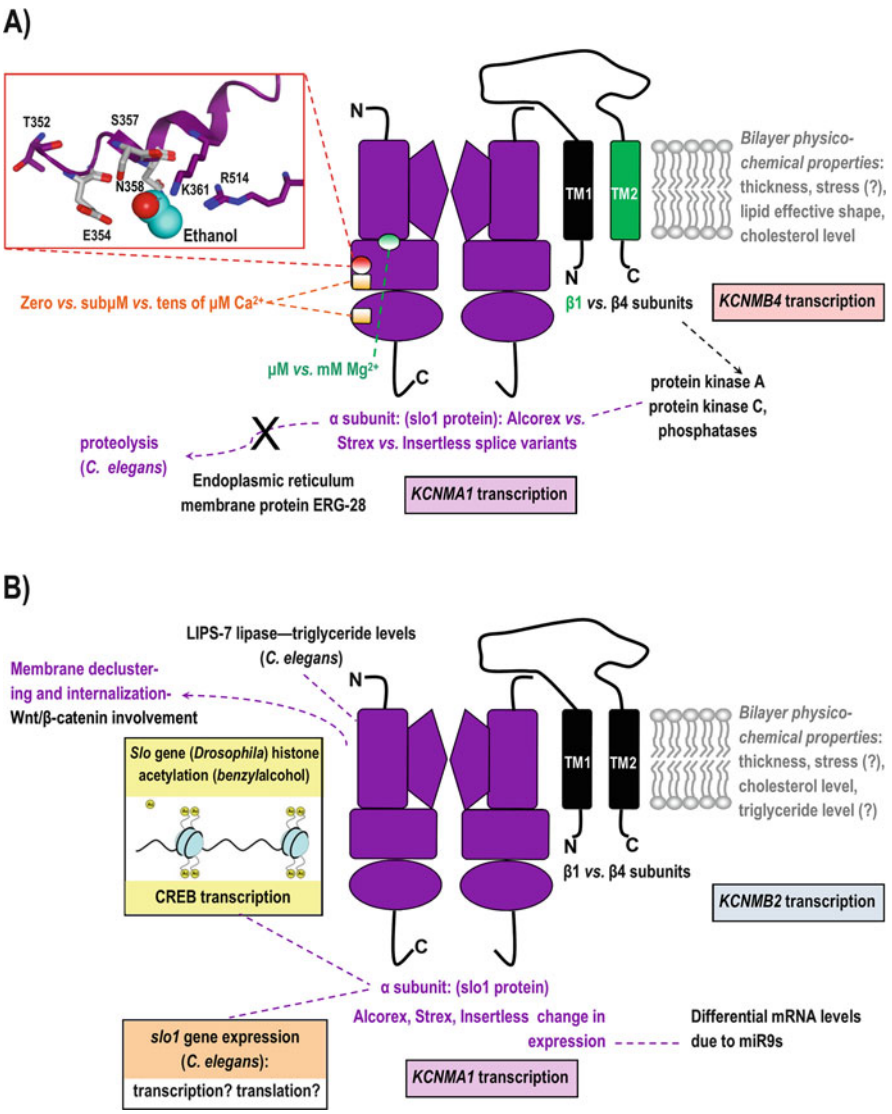

Fig. 2 Molecular determinants of the functional interaction between BK and alcohol: (**a**) in alcohol-naïve systems following single and brief (min) alcohol exposure; (**b**) during protracted (hours to days) or repeated alcohol administration, which leads to different forms of tolerance to alcohol action. Tolerance was determined by electrophysiological, biochemical, genetic, and/or behavioral assays. While different splice variants of slo1 show different electrophysiological responses to acute ethanol exposure, the differential expression of slo1 variants secondary to miR9s only occurs upon protracted ethanol. BK β1 TM2 determines a unique response of recombinant slo1 + β1 heteromers to acute ethanol exposure, i.e., decrease in activity under conditions of voltage and $Ca^{2+}{}_i$ that are physiological in the contracting cerebral artery myocyte. It remains unknown, however, whether this channel region plays a role in the ethanol response of β1-containing BK in native, *neuronal* channels

eliminate the development of AFT, indicating that *slo-1* is involved in, but is not required for, AFT to ethanol (Bettinger et al. 2012). In addition, *slo-1* plays an important part in the response of *C. elegans* to longer treatment with ethanol; when *C. elegans* are exposed to a sedating dose of ethanol for 18–24 h and then withdrawn, they demonstrate behavioral impairments (Davies et al. 2004; Mitchell et al. 2010; Scott et al. 2017). The depression of locomotion that is associated with withdrawal is enhanced in *slo-1* mutant animals, and animals carrying a *slo-1* gain-of-function mutation display attenuated withdrawal (Scott et al. 2017). A 24-h exposure to a sedating dose of ethanol decreased the intensity of the signal from a *slo-1*:: *fluorescent reporter* transgene chimera in some neurons, suggesting that ethanol might induce a reduction in *slo-1* expression. The mechanism of this regulation (transcriptional or posttranscriptional), however, remains to be established (Scott et al. 2017).

The role of BK in behavioral tolerance to ethanol has been extensively studied in *Drosophila melanogaster*. In a rapid tolerance assay, *Drosophila* were exposed to a sedating dose of ethanol, removed from the ethanol, and the time for them to recover was recorded. After this exposure, flies developed rapid tolerance, such that when they were sedated a second time, their recovery was faster (Cowmeadow et al. 2005). *Slo* mutant animals did not develop rapid tolerance to ethanol, demonstrating a central role for BK in this process (Cowmeadow et al. 2005). Transcriptional regulation underlies the *slo*-dependent development of rapid tolerance in *Drosophila*: ethanol sedation increased *slo* expression, and artificial induction of *slo* expression decreased ethanol sensitivity, a phenotype that resembled rapid tolerance to ethanol (Cowmeadow et al. 2006). This ethanol-induced change in *slo* transcription was dependent on modulation of histone acetylation in the *slo* promoter (Wang et al. 2007). Moreover, both CREB and CBP were required for this effect on *slo* expression (Wang et al. 2009; Ghezzi et al. 2017).

4.2 Modifiers of BK-Mediated Acute and Rapid Tolerance to Ethanol: BK Accessory Subunits, Transcriptional Regulation, Posttranslational Modifications, and Lipid Metabolism/Levels

4.2.1 BK Accessory Subunits

BK also plays a role in acute behavioral responses to ethanol including the development of tolerance in mammals. Several studies have directly tested the role of BK function in the behavioral responses to alcohol using a mouse KO approach. While experiments examining the function of BK α have not been reported, the β1 and β4 subunits have both been examined. Knockout of either β1 or β4 did not alter the acute effects of ethanol in ethanol-naïve animals, as tested by rotorod at 1.5 g/kg ethanol, loss of righting reflex (LORR) at 4 g/kg, and body temperature over 2 h at 4 g/kg (Kreifeldt et al. 2015). However, both β subunits affected different aspects of the responses to chronic ethanol treatment: after *wt* animals were exposed to chronic intermittent ethanol (CIE), they developed

tolerance to the sedating effects of ethanol as tested by the LORR assay and to ethanol-induced hypothermia. β1 KO but not β4 KO animals demonstrated a decrease in the development of tolerance to both of these effects of ethanol (Kreifeldt et al. 2015), unveiling a role for β1 in the development of tolerance. In contrast, both subunits were involved in a different measure of the response to chronic ethanol treatment. After CIE, animals were more sensitive to demonstrating withdrawal-induced hyperexcitability, which was measured by the susceptibility of animals to demonstrate handling-induced convulsions (HIC). β1 and β4 KO affected different aspects of HIC: β1 KO increased HIC at 6 h post-ethanol injection whereas β4 KO increased HIC at 12 h post-ethanol injection, which suggests that β1 is involved in the appearance of withdrawal-induced hyperexcitability whereas β4 has a role in its resolution (Kreifeldt et al. 2015).

β4 was also implicated in modulating the chronic effects of ethanol in a separate series of experiments. When control animals were injected with 2 g/kg of ethanol, their locomotion was suppressed at 5, 10, and 15 min postinjection. After 4 days of treatment with 2 g/kg ethanol, the animals were equally sedated at 5 and 10 min postinjection but had developed the ability to demonstrate acute functional tolerance to this effect at 15 min postinjection. β4 KO mice had a strikingly different phenotype; on the first day, while being significantly sedated by ethanol at 5 min postinjection, animals displayed a robust acute functional tolerance to the sedating effects of ethanol at 10 min postinjection. After 4 days of treatment, the development of tolerance was significantly enhanced in β4 KO mice relative to *wt* (Martin et al. 2008). These data indicate that BK β4 is central in the development of AFT to ethanol in mammals and, importantly, that this role of BK β4 can be modulated over repeated ethanol exposures.

4.2.2 Transcriptional Regulation

The transcriptional changes elicited by a 4 h exposure of the brain to ethanol were globally assessed in C57BL/6J and DBA/2J mice (Kerns et al. 2005) and in a set of mouse strains derived from these inbred strains (Wolen et al. 2012). Three brain regions were studied: nucleus accumbens, prefrontal cortex, and ventral midbrain. In each region, *KCNMA1* was found to be transcriptionally regulated in response to ethanol exposure (Kerns et al. 2005; Wolen et al. 2012). In the ventral midbrain, *KCNMB4* was also regulated with ethanol exposure (Wolen et al. 2012). Thus, transcriptional regulation may constitute a widespread mechanism by which BK function is changed in response to ethanol exposure.

This same series of recombinant inbred mouse lines were used to directly test the hypothesis that *KCNMA1* expression was altered with chronic ethanol exposure (Rinker et al. 2017). Transcript levels from the nucleus accumbens and prefrontal cortex of animals chronically exposed to ethanol in either the forced CIE paradigm or the voluntary two-bottle choice paradigm were examined. *KCNMA1* mRNA levels were significantly correlated with both voluntary consumption and CIE in both brain regions (Rinker et al. 2017). Animals that had undergone the CIE treatment became ethanol-dependent and increased their voluntary ethanol consumption. In the prefrontal cortex, transcript levels of *KCNMB2* were significantly

correlated with dependence-induced change in voluntary drinking, further connecting transcriptional regulation of BK function with ethanol drinking (Rinker et al. 2017).

Although there is a single BK α encoding gene in mammals, there is a rich diversity of BK isoforms that is achieved through alternative splicing of BK mRNAs. The different splice forms code for slo1 channels with different phenotypes, including drastic differences in their ethanol sensitivity (Pietrzykowski et al. 2008). In rat supraoptic nucleus cells, the microRNA miR-9 was rapidly upregulated in response to ethanol exposure and, in turn, decreased expression of particular BK isoforms through selectively destabilizing their mRNAs with antisense complementarity to miR-9 in their 3'UTRs. This isoform-specific regulation changes the BK population in the neuronal cells to strongly favor those isoforms that are less or not sensitive to alcohol, which is predicted to dramatically decrease the ethanol sensitivity of the overall BK current in response to alcohol (Pietrzykowski et al. 2008).

4.2.3 Posttranslational Modifications

The effects of ethanol exposure on the physiological aspects of BK channel function have been extensively studied in rat neurohypophysial terminals (see Sect. 3.4). In particular, in rats that had been exposed to ethanol for 3 weeks, BK currents were less sensitive to activation by ethanol and demonstrated lower current density, suggesting that they had developed tolerance to the drug (Knott et al. 2002). Tolerance that developed over 24 h of ethanol exposure was found to be intrinsic to the tissue that is targeted by ethanol and had two phases: a fast decrease in ethanol potentiation of the BK current that was detectable by 12 min of exposure to ethanol, representing acute molecular tolerance; this effect was maintained for at least 24 h. In addition, a second, distinct response to longer-term treatment with ethanol was identified; after 24 h of ethanol exposure, BK current density was decreased. This decrease in current density was coincident with a decrease in BK clustering and a movement of channels away from the plasma membrane (Pietrzykowski et al. 2004). Indeed, upon careful analysis, the subcellular localization of BK channels in ethanol-exposed neurons was found to be dynamic: in rat primary hippocampal neuron preparations, upon exposure to ethanol, there was a transient increase in BK channels on the cell surface that lasted for 3 h. Importantly, during the transient increase in the BK population on the cell surface, there was a decrease in BK function, providing evidence that this ethanol-induced change in localization is not the only mechanism underlying the change in BK function in response to ethanol exposure (Palacio et al. 2015). After 6 h of exposure to ethanol, however, BKs were internalized and removed from the plasma membrane, whereas the total BK protein level remained unchanged (Palacio et al. 2015). This was consistent with the previous observation that 6 h of ethanol exposure caused a decrease in channel number in rat neurohypophysial membranes (Pietrzykowski et al. 2004). This persistent decrease in slo1 channel number on the cell surface after 6 h of ethanol treatment can be inhibited by inhibiting protein synthesis in cultured rat hippocampal neurons (Velázquez-Marrero et al. 2016). Since the total BK protein level is unchanged after 6 h of ethanol exposure (Palacio et al. 2015; Velázquez-Marrero et al. 2016), the protein synthesis that is required for BK

internalization must be producing other proteins that act in this process. Levels of β-catenin, known to be important in BK surface expression (Bian et al. 2011), were increased in response to ethanol treatment, and the Wnt/β-catenin signaling pathway was found to be required for the redistribution of BK after 6 h of ethanol treatment (Velázquez-Marrero et al. 2016).

4.2.4 Lipid (Cholesterol-Triglyceride) Metabolism/Levels

In artificial electrophysiological preparations, the composition of the lipid bilayer strongly modulates the ability of the human slo1 channel to develop tolerance to ethanol. BKs are activated by ethanol, and within 10 min they display tolerance to this effect. In artificial lipid bilayers, when the bilayer thickness was modified by changing the lipid composition, the ability of ethanol to activate BK, as well as the ability of BK to develop acute tolerance, could be modified, demonstrating a central role for lipid milieu in the function of BK and the effects of ethanol on that function (Yuan et al. 2008).

Behavioral studies in *C. elegans* support the in vitro data documenting that BK interaction with the lipid milieu is an important regulator of BK in vivo function, including the BK response to ethanol. Cholesterol is an important component of the lipid bilayer. *C. elegans* derive cholesterol exclusively from their diet, so it can be depleted experimentally. Cholesterol-starved *C. elegans* were unable to develop AFT to ethanol, demonstrating that there is an absolute requirement for cholesterol in this form of tolerance (Bettinger et al. 2012). Furthermore, animals carrying a mutation in the triacylglyceride lipase *lips-7* had higher lipid levels than *wt*, were resistant to ethanol, and demonstrated enhanced development of AFT to the alcohol. Importantly, *lips-7* and *slo-1* showed an intriguing genetic interaction: *lips-7* mutant animals had a locomotion phenotype consistent with that of a loss-of-function *slo-1* mutant, and the *lips-7* mutation could significantly suppress the depressed locomotion phenotype of *slo-1* gain-of-function mutations. These data demonstrate that the function of this triacylglyceride lipase is important for the modulation of SLO-1 function (Bettinger et al. 2012).

4.3 Putative Role of BK in Alcohol Consumption and Alcohol Use Disorders

BK has well-established roles in the acute behavioral responses to ethanol across the phylogenetic spectrum, which suggests that BK may play a role in more complex behavioral responses to ethanol. The effect of manipulating BK function on voluntary ethanol consumption was tested in mice. Beta4 KO mice were found to consume significantly more alcohol and to achieve a significantly higher blood alcohol concentration in the drinking in the dark paradigm than *wt* (Martin et al. 2008). These data strongly indicate that BK activity is an important modulator of biologically relevant vertebrate ethanol response behaviors.

The finding that repeated exposures to ethanol could modify the effects of BK on a tolerance phenotype suggests that modulation of BK function may play a role in the behavioral response to ethanol in animals that had had prior exposure to the

drug. When *wt* mice were made physically dependent on ethanol in the chronic intermittent exposure paradigm, and then subsequently allowed only limited voluntary access to alcohol, they consumed more alcohol over time (Kreifeldt et al. 2013). Intriguingly, β1 and β4 KO mice had significantly different behavioral responses to alcohol under this paradigm: β1 KO animals demonstrated enhanced escalation of voluntary consumption compared to *wt*. In contrast, β4 KO animals had attenuated escalation of drinking relative to *wt* (Kreifeldt et al. 2013). These data clearly indicate that BK activity plays an important part in voluntary ethanol consumption.

The effects of changing BK function in voluntary alcohol consumption behaviors in mice suggest that BK function in humans is a good candidate for being involved in alcohol drinking. In humans, the propensity to develop an AUD is strongly influenced by genetics (Verhulst et al. 2014), and the identification of specific genetic factors that predispose particular individuals to AUD has been the focus of much study. While it has been difficult to unambiguously identify variants that alter risk for AUD, variations in *KCNMA1* have been repeatedly implicated in several studies conducted in different human populations.

Single nucleotide *KCNMA1* polymorphisms were first identified by Schuckit et al. (2005) as being implicated in the level of response to alcohol demonstrated in a laboratory alcohol challenge. In this study of pairs of siblings of alcohol-dependent parents, authors identified a linkage peak for association with the level of response to alcohol; *KCNMA1* was under the peak, and 44 polymorphisms in *KCNMA1* were directly examined for association with level of response to alcohol. None of the polymorphisms in any gene under examination reached genome-wide significance, but six of the examined *KCNMA1* polymorphisms were nominally associated with the level of response to alcohol. In a separate population assessed for symptoms of alcohol dependence in a questionnaire, *KCNMA1* variation was identified as being associated with symptoms of dependence (Kendler et al. 2011). None of the polymorphisms tested in this study was significantly associated with alcohol dependence symptoms, but a polymorphism in *KCNMA1* was the most significant result. Bolstering this observation, several other *KCNMA1* polymorphisms were nominally associated in this population. Collectively these two studies indicate that multiple polymorphisms in *KCNMA1* show association with alcohol phenotypes.

Examining the Collaborative Study on the Genetics of Alcoholism (COGA), Edenberg et al. (2010) found two additional lines of evidence for the importance of *KCNMA1* in alcohol phenotypes in humans. One polymorphism in *KCNMA1* was one of six that was identified both in European American and African American samples in the COGA case-control sample. Additionally, a polymorphism in *KCNMA1* was nominally associated in the COGA family sample with early onset of alcohol dependence. Han et al. (2013) approached this question somewhat differently and combined different human genome-wide association studies with functional analysis of the most highly associated genes to generate a functional network of genes that they hypothesized were particularly likely to play roles in risk for alcohol dependence. Both *KCNMA1* and, intriguingly, *KCNMB1* were among the 39 genes identified in this study.

4.4 Extrapolations of BK Genetic Studies on Ethanol Use Disorders Between Invertebrate Organisms, Rodents, and Humans

Studies in model organisms including both invertebrate and vertebrate systems have consistently demonstrated that BK plays important roles in ethanol response behaviors across phyla. In both *C. elegans* and mouse, there is ample electrophysiological evidence that ethanol acts directly on BK proteins, and the behavioral evidence demonstrates that this action of ethanol has important behavioral consequences (see above). Genes that modulate the acute level of response to alcohol in invertebrates are excellent candidates for having roles in alcohol dependence in humans (Grotewiel and Bettinger 2015). In mouse, the behaviors that BK mediates include both acute levels of response to alcohol and, importantly, voluntary consumption of alcohol. Interestingly, altering the function of different BK subunits can increase or decrease alcohol consumption (Kreifeldt et al. 2013), suggesting that different kinds of genetic variation in the BK system may influence risk for alcohol dependence in humans differently. These observations may also imply that genetic variation in BK β subunit genes may also be important in risk for ethanol dependence.

It has been difficult to identify robust signals in human studies of variation in genes that are strongly associated with liability to develop alcohol dependence. This is likely to be due to the complex genetic architecture of alcohol dependence in humans (Kendler et al. 2012) and the relatively small contribution of any individual variant, which will require very large genome sizes to detect. One reason for the small effect of specific variants may be that strong perturbations of function of important ethanol response mediators like BK may not be maintained in human populations. KO of slo1 in mice causes significant behavioral deficits (Typlt et al. 2013). Therefore, it may be that variation tolerated in the human population consists of mild change-of-function alleles, which may be more difficult to detect in human association studies. However, signals that appear in multiple studies, even when not significant in any individual study, are likely to identify genes that are relevant for alcohol dependence phenotypes. While none of the human genome studies individually yielded genome-wide significance for *KCNMA1*, the consistency of identifying variation in *KCNMA1* across many different studies (Schuckit et al. 2005; Edenberg et al. 2010; Kendler et al. 2011; reviewed by Rinker and Mulholland 2017) points very strongly to the importance of this gene in alcohol dependence phenotypes. Furthermore, the finding of association with *KCNMB1* (Han et al. 2013) provides additional support to a central role for BK function in human alcohol dependence.

5 Conclusion

In summary, BK is one of the central regulators of alcohol's effects in the brain. Although the sensitivity of BK to clinically relevant concentrations of alcohol was described nearly two decades ago, new concepts and pathways of interference with

alcohol-BK interactions are emerging rapidly. Some of the most recent findings include work on epigenetic modifications over gene expression of slo1 proteins and its accessory subunits, as well as cellular trafficking mechanisms that are targeted by both acute and protracted alcohol exposures. The fundamental role played by BK in neuronal physiology spans from invertebrate to mammalian species and, thus, bolsters the applicability of findings in animal models to the effects of alcohol intoxication and AUD pathophysiology in humans. However, bedside interventions that would target alcohol-BK interaction(s) in the central nervous system are yet to emerge.

Acknowledgments This work was supported by the National Institute of Alcohol Abuse and Alcoholism through grants R37-AA11560 (AD), R01 AA-023764 (AB), and R01 AA-024482 (JB).

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Chronic Alcohol, Intrinsic Excitability, and Potassium Channels: Neuroadaptations and Drinking Behavior

Reginald Cannady, Jennifer A. Rinker, Sudarat Nimitvilai, John J. Woodward, and Patrick J. Mulholland

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The original version of this chapter was revised. A correction to this chapter is available at https://doi.org/10.1007/164_2018_192.

R. Cannady · J. A. Rinker · S. Nimitvilai · J. J. Woodward · P. J. Mulholland (✉)
Departments of Neuroscience and Psychiatry and Behavioral Sciences, Charleston Alcohol Research Center, Addiction Sciences Division, Medical University of South Carolina, Charleston, SC, USA
e-mail: mulholl@musc.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_90

Abstract

Neural mechanisms underlying alcohol use disorder remain elusive, and this lack of understanding has slowed the development of efficacious treatment strategies for reducing relapse rates and prolonging abstinence. While synaptic adaptations produced by chronic alcohol exposure have been extensively characterized in a variety of brain regions, changes in intrinsic excitability of critical projection neurons are understudied. Accumulating evidence suggests that prolonged alcohol drinking and alcohol dependence produce plasticity of intrinsic excitability as measured by changes in evoked action potential firing and after-hyperpolarization amplitude. In this chapter, we describe functional changes in cell firing of projection neurons after long-term alcohol exposure that occur across species and in multiple brain regions. Adaptations in calcium-activated ($K_{Ca}2$), voltage-dependent (K_V7), and G protein-coupled inwardly rectifying ($K_{ir}3$ or GIRK) potassium channels that regulate the evoked firing and after-hyperpolarization parallel functional changes in intrinsic excitability induced by chronic alcohol. Moreover, there are strong genetic links between alcohol-related behaviors and genes encoding $K_{Ca}2$, K_V7, and GIRK channels, and pharmacologically targeting these channels reduces alcohol consumption and alcohol-related behaviors. Together, these studies demonstrate that chronic alcohol drinking produces adaptations in $K_{Ca}2$, K_V7, and GIRK channels leading to impaired regulation of the after-hyperpolarization and aberrant cell firing. Correcting the deficit in the after-hyperpolarization with positive modulators of $K_{Ca}2$ and K_V7 channels and altering the GIRK channel binding pocket to block the access of alcohol represent a potentially highly effective pharmacological approach that can restore changes in intrinsic excitability and reduce alcohol consumption in affected individuals.

Keywords

After-hyperpolarization · Alcohol drinking · Alcohol use disorder · Intrinsic excitability · Potassium channels

1 Introduction

The term addiction is commonly used to denote an unnatural or unbalanced amount of attention directed toward an object, substance, or activity. In clinical terms, the term addiction has largely been replaced by the phrase "use disorder" that reflects the understanding that these conditions are diseases or illnesses of the brain that result from some underlying alteration in normal physiology. Substance use disorders (SUDs) and more specifically those involving alcohol (ethanol) are operationally defined as the inability of the individual to control or reduce drug or alcohol intake even in the face of negative social, emotional, or health-related consequences (DSM-5 2013).

An emerging theme in the study of drug and alcohol addiction is that different neural systems and neurochemicals underlie various stages associated with drug and alcohol intake that contribute toward the progression of a drug-/alcohol- dependent state (Koob 2013). In one such model, a binge/intoxication stage is thought to

involve midbrain dopamine, opioid, and endocannabinoid reward pathways that are activated by acute ingestion of drugs/alcohol and induce positive and pleasurable feelings. Metabolic clearance of the drug or alcohol then induces a stage of acute withdrawal that may engage brain stress systems involving hypothalamic and extrahypothalamic circuits leading to negative or unpleasant symptoms that drive additional binge/intoxication stages. The desire to retake a drug or alcohol following some period of abstinence represents the preoccupation/anticipation stage and is associated with feelings of craving characterized as a need to seek out and consume the substance. This phase is thought to reflect activity in higher cortical areas including areas of the prefrontal and orbitofrontal cortex that are involved in guiding behavior by assessing risk and reward associated with making choices between competing activities.

While the brain circuitry underlying these three phases of the cycle is evolutionarily adapted for pursuing natural rewards, repeated episodes of drug or alcohol consumption may lead to an imbalance between these systems with reductions in reward signaling and altered activity in brain stress and preoccupation/anticipation pathways that combine to produce escalations in drug/alcohol use and the development of an SUD. The yearly incidence for developing an alcohol use disorder (AUD) varies among different populations and is approximately 6% for US adults age 18 and older (Substance Abuse and Mental Health Administration (SAMHSA) 2015). Multiple factors enhance an individual's susceptibility for developing an AUD including genetic and epigenetic mechanisms that influence the sensitivity of brain reward, stress, and cognitive systems to alcohol. Alcohol dependence disrupts systems designed to maintain and regulate excitability, and in this chapter, we focus on the role of three subtypes of K^+-selective ion channels ($K_{Ca}2$, K_V7, and $K_{ir}3$ channels) in regulating intrinsic excitability and discuss how alcohol exposure alters the expression and function of these channels. We also highlight findings from recent studies showing how manipulating these channels with selective pharmacological agents can moderate alcohol consumption and facilitate extinction of cue-induced alcohol-seeking behaviors.

2 Alcohol and Intrinsic Excitability

Although several types of cells including those of skeletal and cardiac muscle are capable of generating action potentials, neurons have exploited this process to generate the complex set of electrical signals that underlie brain function. Action potentials reflect the opening of sodium permeable ion channels activated during periods of membrane depolarization elicited by excitatory synaptic inputs. The rapid entry of positively charged sodium ions drives the membrane potential toward the sodium equilibrium potential that is approximately +40 mV for most mammalian neurons. Voltage-dependent K^+ ion channels are activated at these highly depolarized potentials and, combined with sodium channel inactivation, repolarize the neuronal membrane. Repolarization is often accompanied by an overshoot called the after-hyperpolarization (AHP) that briefly drives the membrane potential past its normal resting value. The AHP helps regulate the frequency of action potential

generation and is mediated by a variety of ion channels including those regulated by changes in intracellular calcium (e.g., $K_{Ca}1$ and $K_{Ca}2$ channels). Neuronal firing is also regulated by activation of channels in the K_V7 family whose activity is normally inhibited by G protein-coupled neurotransmitter receptors and by inwardly rectifying K^+ channels ($K_{ir}3$) that are activated by G proteins (also called GIRK channels).

Intrinsic excitability is a measure of a neuron's ability to fire and is experimentally determined by measuring the number of action potentials generated spontaneously or during a series of current pulses delivered to the neuron using the current-clamp mode of whole-cell electrophysiology. Importantly, plasticity of intrinsic excitability is a critical mechanism underlying synaptic integration and learning processes (Sehgal et al. 2013), and alterations in cellular firing by abused substances may facilitate drug-induced pre- and postsynaptic adaptations (Kourrich et al. 2015). The following section briefly reviews studies that have examined the effects of acute and chronic alcohol exposure on the firing properties of neurons (for review, see Harrison et al. 2017). Later sections focus on how alcohol-induced changes in the expression or function of $K_{Ca}2$, K_V7, and GIRK channels may underlie changes in the current-spike relationship, thus altering the intrinsic excitability of neurons.

2.1 Acute Alcohol and Spike Firing

Brain slice recordings have revealed that under basal conditions, neurons in ventral tegmental area (VTA), globus pallidus, cerebellum, and lateral habenula (LHb) are spontaneously active, while those from dorsal striatum, nucleus accumbens (NAc), hippocampus, and most cortical areas are silent although firing can be induced by direct current injection. Acute application of relatively high concentrations of alcohol (~40–80 mM) enhances firing of dopamine VTA neurons (Brodie et al. 1999), while a lower concentration (20 mM) was shown to increase firing of DA neurons located in the medial VTA (Mrejeru et al. 2015). Concentrations of alcohol as low as 1 mM increased spontaneous firing of glutamatergic neurons in the LHb, while 50 mM alcohol increased the frequency of action potential spiking in cerebellar Golgi neurons (Carta et al. 2004). In contrast, alcohol (10–80 mM) inhibits spontaneous firing of low-frequency (<30 Hz) globus pallidus neurons while having no effect on those that fire at higher frequencies (Abrahao et al. 2017). This effect was occluded by a blocker of $K_{Ca}1$ channels that are a known target for alcohol (Mulholland et al. 2009). Studies using current-evoked spiking report that alcohol also reduces firing of GABAergic neurons in the VTA (Gallegos et al. 1999), pyramidal neurons in the lateral orbitofrontal cortex (lOFC) (Badanich et al. 2013; Nimitvilai et al. 2016, 2017a), and serotonergic neurons of the dorsal raphe (Maguire et al. 2014). Alcohol inhibition of VTA GABAergic neuron firing may involve α6-containing nicotinic acetylcholine receptors (Schilaty et al. 2014), while the alcohol-induced reduction in the firing of lOFC and dorsal raphe neurons requires activation of strychnine-sensitive glycine receptors (Badanich et al. 2013; Maguire et al. 2014).

2.2 Chronic Alcohol and Spike Firing

Several studies have reported changes in intrinsic excitability following chronic exposure to alcohol (Table 1). Spike firing was increased in NAc medium spiny neurons (MSNs) recorded from rats following operant self-administration of alcohol (Hopf et al. 2010), and this was accompanied by reduced AHP amplitude and function of apamin-sensitive $K_{Ca}2$ channels. Similarly, repeated systemic alcohol treatment and long-term drinking in an intermittent access model increased spontaneous action potentials and reduced AHP amplitude in LHb neurons (Agrawal et al. 2012). In contrast, no changes in spike firing or $K_{Ca}2$ channel currents were reported for NAc MSNs from rats given repeated oral doses of alcohol (Marty and Spigelman 2012). Following repeated cycles of chronic intermittent ethanol (CIE) vapor exposure in mice, spike firing of NAc medium spiny neurons and lOFC pyramidal neurons was increased along with reduced AHP and loss of apamin-sensitive currents (Nimitvilai et al. 2016; Padula et al. 2015). Renteria and colleagues reported that the increased firing after CIE exposure was observed only in D1R-expressing MSNs in the NAc shell (Renteria et al. 2017). The increase in spike firing in lOFC neurons persisted for up to 10 days and was associated with a loss of monoamine (DA, NE, 5HT) and GIRK channel modulation of firing and a tolerance to the acute inhibitory actions of alcohol (Harrison et al. 2017; Nimitvilai et al. 2016, 2017b). A similar attenuation of alcohol's inhibitory effect on spike firing was observed in lOFC neurons from long-term drinking (~9 months) monkeys, while current-evoked spiking was reduced in these animals (Nimitvilai et al. 2017a). In contrast, intrinsic excitability of putamen MSNs was increased in monkeys following prolonged drinking and repeated periods of abstinence (Cuzon Carlson et al. 2011). Region-specific changes in spike firing following CIE treatment of mice have also been reported with increases for neurons in the ventral BNST (Marcinkiewcz et al. 2015; Pleil et al. 2015) and dorsal raphe (Lowery-Gionta et al. 2015), decreases in medial central nucleus of the amygdala (Pleil et al. 2015), and no change in infralimbic cortex (Pleil et al. 2015).

One key factor to consider from these types of studies is the time of recording relative to the last alcohol exposure. For example, in the mouse lOFC studies, CIE-exposed animals showed elevated spike firing by the 3-day withdrawal period, and excitability was still elevated at the 10-day withdrawal time point (Nimitvilai et al. 2016), while no change in excitability was seen in animals undergoing acute (<6 h) withdrawal (Nimitvilai et al. 2017a). Studies examining chronic alcohol-induced changes in intrinsic excitability in prelimbic cortical pyramidal neurons are inconsistent, with two studies showing no alterations in evoked firing in layer V neurons and one study reporting an increase in excitability of layer II/III neurons (Pleil et al. 2015; Hu et al. 2015; Trantham-Davidson et al. 2014). Changes in NAc neuron excitability in alcohol-exposed mice and rats were observed during early withdrawal (3–7 days; Padula et al. 2015) as well as following extended abstinence (Hopf et al. 2010). The increase in dorsal raphe neuron spiking following CIE exposure in mice was present 24 h after the last treatment but was lost after 1 week of withdrawal (Lowery-Gionta et al. 2015). Overall, these findings highlight

Table 1 Chronic alcohol effects on intrinsic excitability, after-hyperpolarization amplitude, and $K_{Ca}2$ currents in rodents and nonhuman primates

Brain region	Alcohol exposure model	AP firing	AHP amplitude	$K_{Ca}2$ amplitude	Species	Reference
BNST	CIE	↑	N/R	N/R	Mouse	Pleil et al. (2015)
BNST	CIE	↑	N/C	N/R	Mouse	Marcinkiewcz et al. (2015)
Central amygdala	CIE	↓	N/R	N/R	Mouse	Pleil et al. (2015)
Dorsal raphe	CIE	↑	N/R	N/R	Mouse	Lowery-Gionta et al. (2015)
Infralimbic cortex	CIE	N/C	N/R	N/R	Mouse	Pleil et al. (2015)
Lateral habenula	Repeated alcohol (IP); IAA drinking	↑	↓	N/R	Rat	Kang et al. (2017)
NAc	Operant self-administration	↑	↓	↓	Rat	Hopf et al. (2010)
NAc	Repeated alcohol (IG)	N/C	↑	N/C	Rat	Marty and Spigelman (2012)
NAc	CIE	↑	↓	↓	Mouse	Padula et al. (2015)
NAc	CIE	↑	↑	N/R	Mouse	Renteria et al. (2017)
Orbitofrontal cortex	CIE	↑	↓	↓	Mouse	Nimitvilai et al. (2016)
Orbitofrontal cortex	22 h drinking for 9 months	↓	N/C	N/R	Monkey	Nimitvilai et al. (2017a)
Prelimbic cortex	CIE	↑	N/R	N/R	Mouse	Pleil et al. (2015)
Prelimbic cortex	CIE	N/C	N/R	N/R	Rat	Trantham-Davidson et al. (2014)
Prelimbic cortex	CIE	N/C	N/R	↓	Mouse	Hu et al. (2015)
Putamen	22 h drinking + repeated abstinence	↑	N/R	N/R	Monkey	Cuzon Carlson et al. (2011)
Ventral tegmental area	Repeated alcohol (IP)	↑[a]	↓	↓	Rat	Hopf et al. (2007)

AP action potential, *AHP* after-hyperpolarization, *BNST* bed nucleus of the stria terminalis, *CIE* chronic intermittent ethanol, N/C not changed, *N/R* not reported, *NAc* nucleus accumbens, *IG* intragastric, *IP* intraperitoneal, *IAA* intermittent access to alcohol

[a]Denotes burst firing increased in alcohol-treated rats

the dynamic nature of processes that regulate the intrinsic excitability of neurons across species and reveal that not all alcohol exposure models produce the same effect on cell firing, even within the same brain region.

3 Potassium Channels

The K^+ channel family is the largest known group of ion channels with at least 79 mammalian K^+ channel genes that encode the wide variety of channel subtypes. K^+ channels are highly conserved and ubiquitously expressed in almost all species (Kuo et al. 2005). Currently, there are several distinct categories of K^+ channels based on the mode of activation and gating, including calcium-activated (K_{Ca}), voltage-gated (K_V), inward rectifier (K_{ir}), and two-pore domain K^+ channels (Table 2). Moreover, alternative splicing and co-assembly of varying K^+ channel subunits add to the diversity in K^+ channel subtypes and function (Zandany et al. 2015; King et al. 2016). Despite the large number of K^+ channel subtypes, all share the common feature of a highly selective K^+ ion-permeable transmembrane pore.

Table 2 HUGO Gene Nomenclature Committee (HGNC) designations with their International Union of Pharmacology (IUPHAR) protein names for the potassium channels discussed in this chapter

Gene	IUPHAR protein name (common name)	Description	Activator	Blockers
Kcnn1	K_{Ca}2.1 (SK1)	Small-conductance, calcium-activated	1-EBIO, CyPPA, chlorzoxazone	Apamin
Kcnn2	K_{Ca}2.2 (SK2)	Small-conductance, calcium-activated	1-EBIO, CyPPA, chlorzoxazone	Apamin
Kcnn3	K_{Ca}2.3 (SK3)	Small-conductance, calcium-activated	1-EBIO, CyPPA, chlorzoxazone	Apamin
Kcnq1	K_V7.1	Voltage-gated, delayed rectifier		XE-991
Kcnq2	K_V7.2	Voltage-gated, delayed rectifier	Retigabine	XE-991
Kcnq3	K_V7.3	Voltage-gated, delayed rectifier	Retigabine	XE-991
Kcnq4	K_V7.4	Voltage-gated, delayed rectifier	Retigabine	XE-991
Kcnq5	K_V7.5	Voltage-gated, delayed rectifier	Retigabine	XE-991
Kcnj3	K_{ir}3.1 (GIRK1)	G protein-gated, inwardly rectifying	ML297	Ba^{2+} (nonselective)
Kcnj6	K_{ir}3.2 (GIRK2)	G protein-gated, inwardly rectifying		Ba^{2+} (nonselective)
Kcnj9	K_{ir}3.3 (GIRK3)	G protein-gated, inwardly rectifying		Ba^{2+} (nonselective)

Also shown are the common protein names and the pharmacological agents used to probe alcohol-related behaviors

Given the high intracellular K^+ concentration compared to that in the extracellular environment, channel opening leads to rapid K^+ ion efflux and hyperpolarization/repolarization of the cell membrane potential (Doyle et al. 1998). K^+ channels thus act as an opposing force to cellular depolarization and excitability or as a modifying factor for the shaping of action potentials. Due to the immense number of K^+ channel subtypes and the limited scope of this chapter, we will highlight only three of the K^+ channel families, the K_{Ca} channels, the K_V channels, and the G protein-activated inward rectifier ($K_{ir}3$) channels (Fig. 1). The structure and function of other K^+ channel families and subtypes not mentioned here have been extensively reviewed by others and are referenced for the reader's consideration (Coetzee et al. 1999; Miller 2000; Gonzalez et al. 2012; Jenkinson 2006; Fonseca 2012; Vandenberg et al. 2015).

There are 12 subgroups (K_V1–K_V12) in the family of voltage-gated K^+ channels. K_V channel subtypes are gated by varying voltage dependencies through a voltage-sensing domain formed by four transmembrane segments (S1–S4) in each channel subunit, while the channel pore is comprised of two other transmembrane segments that form a loop (S5–S6). K_V1, K_V4, and K_V7 channels are activated at relatively low membrane potentials and are critical in regulating the number of action potentials during early phases of membrane depolarization (Johnston et al. 2010). The transient A-type current is generated by K_V4 channels and has a unique feature

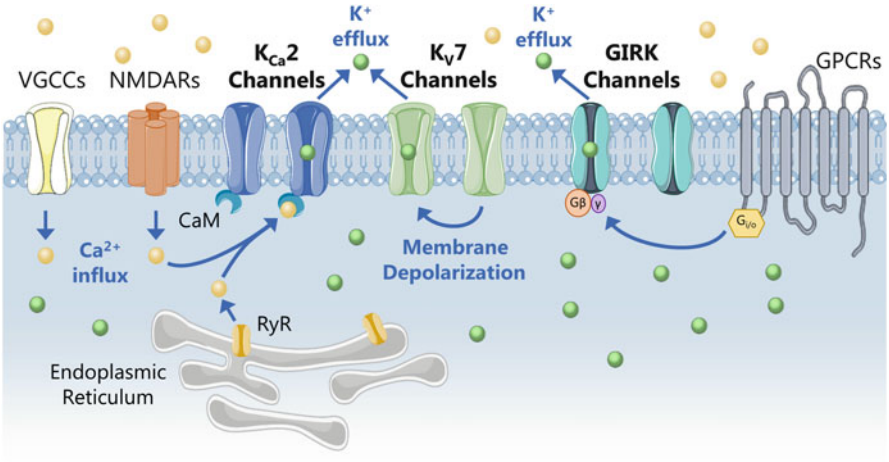

Fig. 1 Activation of $K_{Ca}2$, K_V7, and GIRK channels in neural membranes. $K_{Ca}2$ channels are activated by elevations in intracellular calcium via influx through voltage-gated calcium channels (VGCCs), NMDA receptors (NMDARs), or release from intracellular stores. K_V7 channels are activated near the resting membrane potential and during membrane depolarization. Ligand binding of G protein-coupled receptors (GPCRs) releases G proteins that bind to and activate GIRK channels. Opening of these $K_{Ca}2$, K_V7, and GIRK channels allows potassium ions to flow outside of the cell causing hyperpolarization and shunting of neuronal excitability. *CaM* calmodulin, *RyR* ryanodine receptors. Images were acquired with permission from www.servier.com and subsequently modified

in which hyperpolarization is necessary for the removal of steady-state inactivation before activation (Maffie and Rudy 2008). K_V7 voltage-gated K^+ channels produce the M-current, a slow-developing, low-voltage activated AHP current that is sensitive to muscarinic receptor activation (discussed in detail below). K_V2 and K_V3 currents are activated at high voltages, particularly during the firing of action potentials, and play an important role in membrane repolarization and regulation of firing in neurons with a fast-spiking phenotype. An important feature that differentiates K_V2 and K_V3 channels is the rate of activation with K_V2 channels showing much slower activation, while K_V3 channels open rapidly contributing to their prominent role in regulating firing of fast-spiking neurons. Interestingly, K_V5, K_V6, K_V8, and K_V9 are commonly named the silent electrical K^+ channel subunits. These subunits do not form functional channels as homomers but readily form heteromers with K_V2 channels with diverse functions within tissue types (Bocksteins 2016). The K_V10–K_V12 channels are referred to as ether-à-go-go (denoted K_V10), ether-à-go-go-related (erg, K_V11), and ether-à-go-go-like (elk, K_V12) and are members of the *KCNH* gene family. Few studies exist that directly characterize the exact function of these channels, but generally, these channels become activated at subthreshold voltages and display highly variable gating kinetics (Vandenberg et al. 2015; Zhang et al. 2009).

K_{Ca} channels are unique in that they are activated by increasing levels of intracellular calcium. Each member of this K^+ channel family is further distinguished by the level of single-channel conductance upon activation (Vergara et al. 1998). Large-conductance calcium-activated $K_{Ca}1$ channels that are also voltage-gated have a single-channel conductance of ~100–300 pS, while intermediate ($K_{Ca}3$; ~20–80 pS) and small ($K_{Ca}2$; ~2–20 pS, Marty and Neher 1985) subtypes have more modest values. As discussed below, small-conductance calcium-activated $K_{Ca}2$ channels are of particular interest due to their critical role in regulating intrinsic excitability in relation to alcohol addiction. Alcohol effects on large-conductance calcium-activated $K_{Ca}1$ channels are discussed in the chapter by Dopico and colleagues in this volume (Dopico et al. 2017).

Inward-rectifying K^+ channels consist of seven subfamilies ($K_{ir}1$–$K_{ir}7$) and are distributed in multiple cell types, including cardiomyocytes, endothelial cells, kidneys, and neurons (for review, see Hibino et al. 2010). Among these, G protein-coupled inwardly rectifying K^+ ($K_{ir}3$ or GIRK) channels are considered as important neuronal regulators and are associated with numerous neuropsychiatric diseases, including schizophrenia (Yamada et al. 2012), depression (Llamosas et al. 2015), epilepsy (Signorini et al. 1997), as well as drug and alcohol abuse disorders (Hill et al. 2003). Unlike K_V and K_{Ca}, GIRK channels are activated by ligand-stimulated G protein-coupled receptors (GPCRs), including dopamine, serotonin, and GABA. Binding of an agonist to the GPCR triggers the dissociation of $G_{\alpha i}$ and $G_{\beta\gamma}$. Once released from its bound $G_{\alpha i}$, the $G_{\beta\gamma}$ dimeric protein can directly activate the GIRK channel (Logothetis et al. 1987; Reuveny et al. 1994) and increase its affinity for the membrane-bound phospholipid-phosphatidylinositol 4,5-bisphosphate (PIP2), which is required to stabilize the open state of the GIRK channel (Huang et al. 1998; Xiao et al. 2003). The activated GIRK channel is now permeable to K^+ ions, leading to hyperpolarization of the cell membrane and inhibition of neuronal activity.

Interestingly, concentrations of alcohol relevant to human consumption (18 mM alcohol or 0.08% blood alcohol level) directly activate GIRK channels independently from GPCR-dependent activation (Bodhinathan and Slesinger 2013; Kobayashi et al. 1999; Lewohl et al. 1999). As many therapeutic drugs that target GPCR-linked GIRK channels have been used to treat a number of neuropsychiatric diseases including alcoholism and alcohol itself directly activates GIRK channels, the role of these channels on neuronal activity and its involvement in alcohol addiction is also addressed in this chapter.

4 Intrinsic Excitability

K^+ channel regulation of cellular excitability is among the most important functions related to proper neuronal activity. Excitability of neuronal activity is regulated in many ways including alteration of resting membrane potential (RMP) and limiting neuronal spike activity. The AHP that follows neuronal spiking is a manifestation of outward K^+ current that regulates subsequent spiking episodes and, thus, establishes firing frequency. In addition, summation of the AHP after bursts of action potentials leads to spike-frequency adaptation that ultimately leads to the inhibition of spiking and is key for protecting neurons from over-excitation and epileptiform activity (Alger and Williamson 1988; Garduno et al. 2005; Schulz et al. 2012). The generation and regulation of action potentials by K^+ channels are critical for neuronal encoding of input- and output-specific information and are mediated by the AHP. Intrinsic excitability is also crucial to processes such as spike timing-dependent plasticity in which changes in the strength of neuronal connectivity rely on the relative timing of synaptic events and back-propagating action potentials and is essential for certain types of learning (Debanne and Poo 2010). Therefore, K^+ channel regulation of excitability has important implications in establishing and mediating learning and memory processes. $K_{Ca}2$, K_V7, and GIRK channels play significant roles in controlling intrinsic excitability by regulating RMP, spike frequency, and the AHP. Their functional roles and importance in alcohol-related neuroadaptations and behaviors are discussed in more detail below.

5 $K_{Ca}2$, K_V7, and GIRK Channel Regulation of Excitability

The small-conductance calcium-activated K^+ ($K_{Ca}2$) channels play a key role in regulating intrinsic excitability and underlie the medium AHP (mAHP) that follows action potentials. *KCNN1–3* genes encode the pore-forming α subunits of $K_{Ca}2.1$, $K_{Ca}2.2$, and $K_{Ca}2.3$ channels that are predominantly expressed in the central nervous system (Stocker 2004). These channels are structurally similar to the K_V channels in that they form tetramers and each α subunit contains six transmembrane regions. $K_{Ca}2$ channels are potently inhibited by the bee venom toxin apamin that produces an increase in cellular excitability and reduction in the amplitude of the mAHP (Blatz and Magleby 1986). Subunits of $K_{Ca}2$ channels contain a calcium-sensitive

calmodulin-binding domain formed by constitutively bound calmodulin (Schumacher et al. 2001; Xia et al. 1998) that senses changes in intracellular calcium concentration arising from activation of various calcium channel subtypes, NMDA receptors, and intracellular stores of the endoplasmic reticulum (Adelman et al. 2012). The increase in intracellular calcium concentration that occurs during membrane depolarization induces a conformational change in the calmodulin-binding domain of the $K_{Ca}2$ channel resulting in activation and K^+ ion efflux (Xia et al. 1998). Thus, $K_{Ca}2$ channels are critical for monitoring and regulating intracellular calcium signaling and action potential generation.

Similar to other voltage-gated K^+ channels, K_V7 channels are tetramers formed by assembly of six transmembrane K_V7 protein subunits ($K_V7.1$–7.5) that are encoded by the *KCNQ* family of genes (*KCNQ1–5*, respectively). $K_V7.1$ subunits are found almost exclusively in cardiomyocytes and do not co-assemble with any of the other K_V7 channel proteins and instead associate with *KCNE* proteins that act as ancillary modulatory subunits (Abbott and Goldstein 2001; McCrossan and Abbott 2004; Roura-Ferrer et al. 2010). The remaining four subunits are expressed throughout the nervous system and form functional homotetramers ($K_V7.2$, $K_V7.4$, and $K_V7.5$) or heterotetramers with $K_V7.3$ (Howard et al. 2007; Jentsch 2000). These channels generate the M-current (I_M), a voltage-sensitive K^+ current so named as it is suppressed by activation of muscarinic receptors (Wang et al. 1998; Shah et al. 2002). Subsequent research has demonstrated that the M-current can also be inhibited by a number of different neurotransmitters/hormones that act on G_q- and G_{11}-coupled receptors, including, but not limited to, $mGlu_{1/5}$, histamine H1, 5-HT2C, substance P, bradykinin, and angiotensin II (Brown and Passmore 2009; Zaika et al. 2006; Marrion 1997). Despite the abundance of $K_V7.2/7.3$ channels and the physiological evidence that these heterotetramers contribute substantially to the native M-current, all K_V7 channels produce M-like currents (Brown and Passmore 2009), with $K_V7.4$-containing channels contributing to M-current in midbrain dopaminergic neurons (Hansen et al. 2008). Although $K_V7.5$-containing channels show a less ubiquitous expression profile, they are nonetheless found in cortical, hippocampal, and striatal tissue (Shah et al. 2002; Schroeder et al. 2000). Regardless of composition, each M-channel subunit contains a voltage-sensor domain (transmembrane domains S1–S4), and the channels are slow-activating starting at subthreshold potentials of around −60 mV. Uniquely, M-channels do not inactivate, thus producing a sustained voltage-dependent outward current that stabilizes the membrane potential in the presence of depolarizing currents, thus regulating neuronal excitability by dampening repetitive or burst firing of action potentials (Jentsch 2000; Brown and Passmore 2009; Brown and Adams 1980). These channels also regulate interspike intervals and contribute substantially in determining whether neurons naturally fire tonically or phasically (Wang and McKinnon 1995; Lawrence et al. 2006). They also contribute to aspects of the AHP differentially depending on the brain region, channel composition, and presence/distribution of other K^+ channels. In CA1 pyramidal neurons, M-channels almost exclusively mediate the mAHP (Gu et al. 2005), though not as much in other cell types (Mateos-Aparicio et al. 2014). In contrast, M-channels have been shown to mediate the fAHP and

sAHP in VTA dopamine neurons (Koyama and Appel 2006). Suppression of the M-current by application of the K_V7 channel blocker, XE-991, diminishes respective AHP components and increases cellular excitability and spike discharge (Brown and Passmore 2009; Gu et al. 2005; Koyama and Appel 2006). Thus, K_V7 channels are an essential regulator of intrinsic excitability, the importance of which is underscored by the finding that a mere 25% reduction in function of $K_V7.2/7.3$ channels induces seizures, as found in cases of benign familial neonatal convulsions caused by *KCNQ2* and *KCNQ3* mutations (Schroeder et al. 1998).

$K_{Ca}2$ and K_V7 channels play complementary roles in regulating neuronal excitability and the AHP. $K_{Ca}2$ channels within dentate gyrus granule cells have been identified as the central contributor to the mAHP and spike-frequency adaptation, while K_V7 channels contribute minimally to the isolated mAHP and spike-frequency adaptation but strongly regulate the action potential threshold by activating at subthreshold potentials (Mateos-Aparicio et al. 2014). This complementary function is critical because dentate granule cell activity can regulate the output of interneurons and pyramidal cells through synaptic activity within hippocampal mossy fibers (Henze et al. 2002), and these channels also function to support stable long-term potentiation through facilitating protein synthesis (Barnes et al. 2010). In contrast, in CA1 hippocampal pyramidal cells, $K_{Ca}2$ channels seem to contribute minimally to the mAHP following action potential bursts, whereas K_V7 channels are the predominant regulators of the mAHP (Chen et al. 2014). Moreover, when K_V7 channel function is inhibited or compromised in CA1 pyramidal cells, $K_{Ca}2$ channels usurp regulation of the AHP and intrinsic excitability and function as a "fail-safe" to limit spike discharge (Chen et al. 2014). This has been proposed to have functional implications for conditions in which K_V7 channel function may be disrupted including hyposmolarity-induced epileptiform seizures (Kobayashi et al. 2008), benign familial neonatal convulsions (Singh et al. 2008), or chronic alcohol exposure (Kang et al. 2017). There is limited understanding of how $K_{Ca}2$ and K_V7 channels may interact in other brain regions outside of the hippocampus. The diverse functional interactions within cellular subtypes of the hippocampus alone suggest that interplay between these channels may have varying influences on neuronal firing and AHP in other brain regions and/or cellular subtypes. Future studies are warranted to determine the full extent of K_V7 and $K_{Ca}2$ channel interactions to regulate cell firing, especially as disruptions in K_V7-$K_{Ca}2$ channel cross talk may influence plasticity of intrinsic excitability and alcohol-seeking behavior.

GIRK channels are comprised of four different subunits, GIRK1 to GIRK4. GIRK1–3 subunits (encoded by *Kcnj3*, *Kcnj6*, and *Kcnj9*, respectively, Table 2) are moderately or highly expressed throughout the brain, while expression of GIRK4 is low and is found only in a few brain regions such as the hypothalamus and cerebellum (Aguado et al. 2008; Karschin et al. 1996; Perry et al. 2008). Functional GIRK channels are heterotetramers of GIRK1/2, GIRK1/3, GIRK1/4 or GIRK2/3 subunits, or homotetramers of GIRK2 subunits (Luscher and Slesinger 2010). GIRK2 contains an endoplasmic reticulum (ER) export signal, enabling this subunit to form either homo- or heterotetramers. GIRK3 does not have an ER signal but contains a lysosomal targeting sequence that promotes degradation of GIRK

channels. GIRK1 has neither an ER nor a lysosomal targeting signal so it must assemble with another GIRK subunit to express on the plasma membrane. GIRK currents are termed "inwardly rectifying" because current passes more easily in the inward direction than in the outward direction. Under physiological conditions where the RMP of neurons is positive to the equilibrium potential for K^+, basal current or agonist-induced GIRK currents show large inward and small outward flow. The outward flow of GIRK-mediated K^+ currents near the RMP hyperpolarizes the membrane potential and thus decreases the excitability of the neuron. Dysfunction of GIRK channels has been implicated in several diseases. For example, loss of GIRK function results in excessive excitability found in epilepsy (Signorini et al. 1997), while over-activation of GIRK can reduce neuronal activity and may trigger cell death as postulated in Down's syndrome and Parkinson's disease, respectively (Patil et al. 1995).

6 $K_{Ca}2$ Channels and Alcohol

6.1 Genetics

AUD is a complex brain disease that likely results from interactions among genetic, epigenetic, and environmental factors that combine to promote heavy alcohol drinking and dependence phenotypes. Meta-analyses and twin studies estimated that 23–79% of the variance in alcohol addiction is heritable (Agrawal et al. 2012; NIAAA 2016). The limited success of the current FDA-approved drugs for treating excessive drinking may reflect the heterogeneous population of those with AUD. As precision medicine approaches are showing efficacy across a number of diseases (i.e., cancer and cardiovascular disease), recent clinical and preclinical studies have searched for genetic mutations as possible pharmacogenetic targets to treat alcohol addiction (Kranzler et al. 2017; Rinker and Mulholland 2017). While not all pharmacogenetic studies to treat alcohol addiction are reproducible (Kranzler et al. 2017), some studies have shown that matching genetic variation to pharmacotherapies can improve relapse rates and heavy drinking days (Kranzler and McKay 2012; Heilig et al. 2011; Sturgess et al. 2011). Thus, advances in precision medicine approaches offer a promising strategy for treating alcohol addiction, and a number of novel targets have been identified from preclinical models of alcohol seeking and dependence (Padula et al. 2015; Rinker and Mulholland 2017; Rinker et al. 2017).

A recent preclinical study employing an integrative functional genomics approach revealed that *KCNN* genes are present in multiple quantitative trait loci (QTL) and gene sets related to alcohol intake and dependence (Padula et al. 2015), including an alcoholism susceptibility QTL on human chromosome 1. Each member of the family of *Kcnn* genes is found in QTLs for alcohol consumption and preference in rats and mice (Padula et al. 2015; Bachmanov et al. 2002; Carr et al. 2003; Foroud et al. 2000; Radcliffe et al. 2004). BXD recombinant inbred (RI) strains of mice are an excellent resource to study the genetic diversity of

alcohol-related phenotypes (Philip et al. 2010). To gain a better understanding of *Kcnn* genes in controlling voluntary alcohol intake, the relationship between *Kcnn* and drinking was explored in BXD RI and their parental strains. Interestingly, *Kcnn3* transcript levels in the NAc correlated negatively with voluntary drinking in a two-bottle choice limited-access and the drinking-in-the-dark model (Padula et al. 2015; Rinker et al. 2017). While there are some potential caveats of using BXD RI strains to study the genetic control of alcohol intake (such as the influence of taste and gene-environment interactions), *Kcnn3* was also identified as a candidate signature gene that associated with alcohol preference in low- and high-alcohol-drinking rat lines (Lo et al. 2016). In alcohol-dependent BXD strains, the negative relationship between *Kcnn3* and alcohol intake became more robust, suggesting that alcohol dependence alters *Kcnn* expression and that high levels of *Kcnn* expression protect against escalation of drinking in dependent mice (Padula et al. 2015). Thus, high expression levels of *Kcnn3* may be a protective factor against developing alcohol addiction and dependence. Although not identified in the NAc, reductions in *KCNN2* expression were reported in the frontal cortex and amygdala of alcohol-dependent individuals (Ponomarev et al. 2012). As described below in detail, studies consistently demonstrate that chronic alcohol exposure reduces $K_{Ca}2$ channel function across multiple preclinical models and in divergent cell types, further demonstrating that *KCNN* is a mediator of voluntary and heavy alcohol drinking. This finding is supported by evidence showing that $K_{Ca}2$ channel positive modulators reduce drinking in rodent models (see below). Lastly, *KCNN* genes are present in QTLs related to all abused substances and are altered in brains of addicts (Padula et al. 2015), suggesting that the *KCNN* family of genes is a common mechanism underlying addiction that spans multiple abused substances (Mulholland et al. 2016).

6.2 Alcohol and $K_{Ca}2$ Channel Function

Alcohol has distinct effects on function and membrane trafficking of $K_{Ca}2$ channels that may underlie some of the neuropathology associated with alcohol addiction (Fig. 2). Early studies in rats demonstrated that 20 weeks of chronic alcohol exposure significantly reduced inhibitory postsynaptic potentials and AHP currents in CA1 pyramidal neurons and dentate gyrus granule cells (Durand and Carlen 1984). Similar effects on $K_{Ca}2$-mediated I_{AHP} were later replicated in CA1 pyramidal neurons from organotypic hippocampal slices continuously exposed to alcohol in vitro (Mulholland et al. 2011). Interestingly, synaptic $K_{Ca}2$ channels are part of a negative feedback loop with NMDA receptors within dendritic spine head nanodomains (Mulholland et al. 2011; Ngo-Anh et al. 2005). Mulholland and colleagues (2011) reported a loss of $K_{Ca}2$ channel control of synaptic NMDA receptor activity after chronic alcohol that paralleled increases in excitotoxicity. This effect was attributed to reduced $K_{Ca}2.2$ subunit expression at synaptic sites, and the hyperexcitability was rescued by treatment with 1-EBIO, a $K_{Ca}2$ channel positive modulator. Together, these data demonstrate a relationship between prolonged alcohol exposure and deficits in hippocampal function that may underlie alcohol-induced cognitive impairments observed in clinical settings (Bartels et al. 2007).

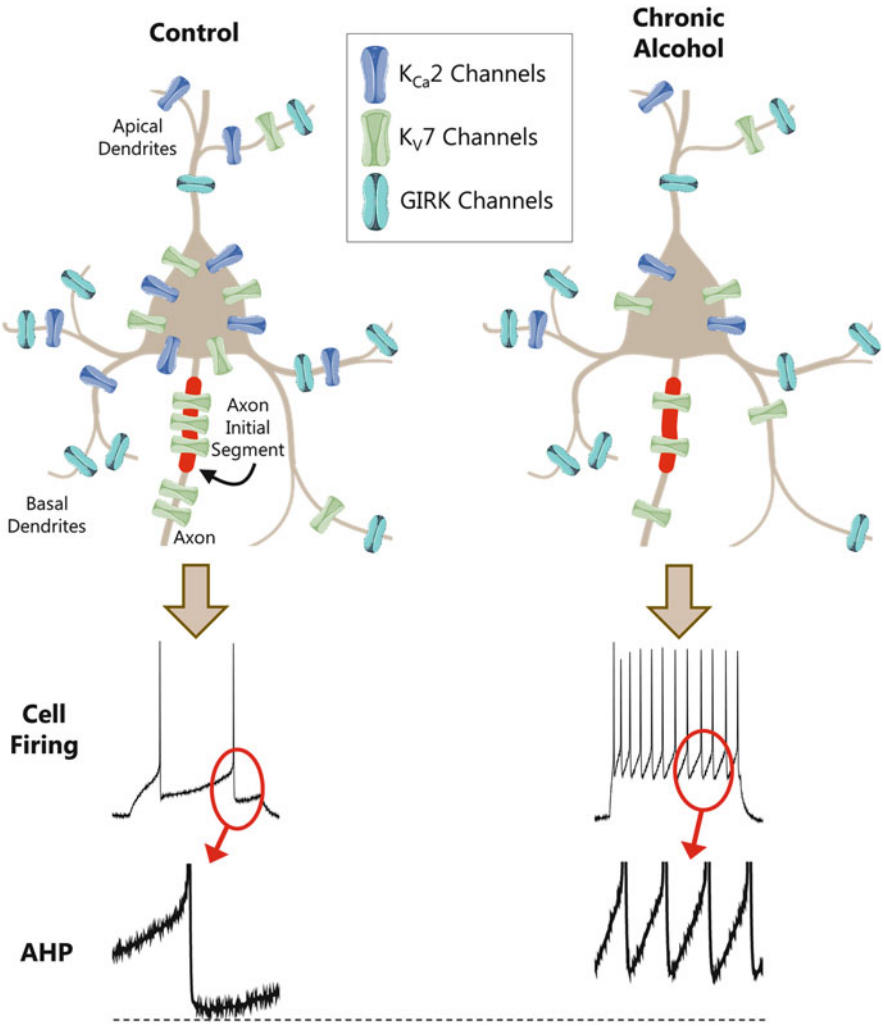

Fig. 2 Chronic alcohol reduces $K_{Ca}2$, K_V7, and GIRK channel expression and signaling in the cortex and striatum. $K_{Ca}2$ and K_V7 potassium channels are expressed in dendrites, axons, and along the soma of neurons where they function to reduce the action potential threshold, increase the after-hyperpolarization (AHP) amplitude, and hyperpolarize the resting membrane potential. GIRK channels localize to synaptic and perisynaptic regions of glutamatergic neurons where they function to dampen neuronal excitation. Chronic alcohol exposure and alcohol dependence reduce expression and function of $K_{Ca}2$ and K_V7 potassium channels in neurons leading to increased intrinsic excitability and reduced AMP amplitude. GIRK channel signaling in the cortex is disrupted in alcohol-dependent mice. Images were acquired with permission from www.servier.com and subsequently modified

Alcohol exposure is also linked to reduced $K_{Ca}2$ channel function within the VTA, a brain region that is the origin of dopamine cell bodies and that plays a significant role in regulating motivation and the rewarding properties of alcohol (Gonzales et al. 2004; Gorelova et al. 2012; Cook et al. 2014). Indeed, studies have shown that bath application of alcohol reduces $K_{Ca}2$ channel function and increases excitability of dopaminergic neurons through modulation of the AHP (Brodie et al. 1999). It was later demonstrated that withdrawal from alcohol reduced AHP and $K_{Ca}2$-mediated I_{AHP} amplitude in VTA neurons (Hopf et al. 2007). Similar effects have also been observed in the NAc and lOFC after induction of alcohol dependence (Nimitvilai et al. 2016; Padula et al. 2015) and voluntary consumption (Hopf et al. 2010). Decreases in NAc $K_{Ca}2$ subunit expression and trafficking also accompany reductions in the AHP amplitude and are associated with increased excitability of the NAc. Interestingly, there was a complete loss of apamin-sensitive I_{AHP} current in NAc and lOFC neurons following chronic alcohol exposure (Nimitvilai et al. 2016; Padula et al. 2015), suggesting that alcohol may affect expression of apamin-sensitive and apamin-insensitive isoforms of $K_{Ca}2.3$ channels (Wittekindt et al. 2004). These data suggest that adaptations in $K_{Ca}2$ channels underlie plasticity of intrinsic excitability and the motivation for alcohol-seeking behavior, particularly during a period of abstinence.

6.3 $K_{Ca}2$ Channel Ligands and Drinking

Several preclinical studies have shown that pharmacological modulation of $K_{Ca}2$ channel activity alters alcohol consumption and seeking behavior. For example, chlorzoxazone is an FDA-approved $K_{Ca}2$ channel activator (Syme et al. 2000; Cao et al. 2001) used as a muscle relaxant that dose-dependently reduced alcohol consumption in rats using the chronic intermittent access drinking protocol (Hopf et al. 2011). The chlorzoxazone-induced reduction in alcohol consumption was associated with the rescue of diminished $K_{Ca}2$ channel activity in the NAc core as examined by ex vivo slice electrophysiology. In addition, infusion of 1-EBIO, a similar $K_{Ca}2$ positive modulator, in the NAc core selectively reduced alcohol, but not sucrose operant self-administration after a period of forced abstinence (Hopf et al. 2010). Using a 24-h intermittent access procedure, investigators were able to show that CyPPA, another $K_{Ca}2$ positive modulator, significantly reduced alcohol consumption in C57BL/6J mice (Padula et al. 2013). Taken together, these preclinical data indicate that treatment with $K_{Ca}2$ positive modulators is a potential promising pharmacotherapeutic approach to reduce alcohol consumption in individuals with alcohol use disorder (Mulholland 2012). Other studies have demonstrated that microinjection of apamin into the NAc significantly increased alcohol consumption in nondependent mice and also induced spontaneous seizure activity in dependent mice at high doses (Padula et al. 2015). These data indicate that there is a bidirectional role for $K_{Ca}2$ channels to modulate alcohol consumption and further emphasize the role of reduced $K_{Ca}2$ channel function in mediating excessive consumption.

In addition to its role in alcohol consumption, $K_{Ca}2$ channels are also involved in regulating responses to alcohol-associated cues. The prefrontal cortex is a key brain region that responds to the presentation of alcohol- and drug-associated cues (Otis et al. 2017), and $K_{Ca}2$ channels within this region have been linked with associative learning processes (Criado-Marrero et al. 2014). Recent evidence has shown that both systemic and local inhibition of infralimbic but not prelimbic cortex $K_{Ca}2$ channels with apamin facilitates extinction of alcohol-seeking behavior during cue extinction sessions (Cannady et al. 2017). This effect is hypothesized to be due to facilitation of newly formed extinction memories to compete with prior cue-associated memories that had once motivated alcohol-seeking behavior. These data demonstrate that $K_{Ca}2$ inhibition could act as a potential therapeutic target to facilitate cue-exposure therapy in problem drinkers.

7 K_V7 Channels and Alcohol

7.1 Genetics

Similar to the relationship between *KCNN* genes and alcohol, integrative functional genomic analysis revealed that *Kcnq2* and *Kcnq3* lie within multiple alcohol-related QTLs in rodents, including those for alcohol consumption and preference (McGuier et al. 2016). Likewise, the remaining members of the *Kcnq* family of genes are present in alcohol preference-related QTLs in rodents (Table 3). In *Drosophila*, low doses of alcohol blocked *KCNQ* currents, and a loss-of-function mutation in *KCNQ* produced an increase in alcohol tolerance and sensitivity to its sedating effects (Cavaliere et al. 2012). *Kcnq2* was identified as a positional candidate within the cis-eQTL for alcohol consumption and withdrawal (Metten et al. 2014), and there

Table 3 GeneWeaver.org gene sets that contain *Kcnq1*, *Kcnq4*, and *Kcnq5* genes in published QTL and curated genomic data sets for alcohol-related behaviors

Gene	GeneWeaver gene set ID	QTL or gene set	Species	PubMed ID
Kcnq1	GS128199	Alcohol preference	Mouse	n/a
	GS84196	Alcohol preference QTL	Mouse	9880655
Kcnq4	GS223356	Alcohol response QTL	Rat	16953387
	GS84162	Chronic alcohol withdrawal severity QTL	Mouse	12925894
Kcnq5	GS84098	Alcohol preference QTL	Mouse	10443995
	GS135279	Alcohol preference QTL, male specific	Mouse	10443995
	GS18838	Differential expression	Rat	12462420
	GS223361	Alcohol response QTL	Rat	15608595
	GS84096	Alcohol consumption QTL	Mouse	11109025
	GS84097	Alcohol preference QTL	Mouse	9880657
	GS84101	Alcohol withdrawal QTL	Mouse	9655868

are two single nucleotide polymorphisms (SNPs) in *Kcnq2* that could account for the differences in drinking phenotypes across multiple mouse crosses (McGuier et al. 2016). In BXD RI strains, *Kcnq* transcript levels in the NAc and prefrontal cortex negatively correlated with voluntary alcohol drinking in a limited-access model (Rinker et al. 2017). Transcript expression levels of genes encoding $K_V7.2$, $K_V7.3$, and $K_V7.5$ channel subunits are altered in key brain regions within the addiction circuitry in postmortem brain tissue from alcoholics and rodent models of chronic alcohol exposure or intake (Rinker and Mulholland 2017; Rinker et al. 2017; McGuier et al. 2016). In the NAc of BXD RI strains, adaptations in *Kcnq1* and *Kcnq5* transcript levels correlated with the change in voluntary drinking in alcohol-dependent mice (Rinker et al. 2017). Moreover, a recent whole genome sequencing study identified *Kcnq5* as a gene associated with alcohol preference in rats (Lo et al. 2016). These preclinical findings are intriguing given that SNPs in *KCNQ1* and *KCNQ5* are associated with early-onset alcoholism and symptoms of alcohol dependence (Kendler et al. 2011; Edenberg et al. 2010). Together, genetic findings linking alcohol action to the family of *KCNQ* genes across species as diverse as fruit flies and humans provide strong evidence that genetic diversity in *KCNQ* influences heavy alcohol intake and contributes to risk factors for developing an AUD.

7.2 Alcohol and K_V7 Channel Function

There is accumulating evidence demonstrating that acute alcohol has direct effects on K_V7 channel activity leading to alterations in the AHP and intrinsic excitability of neurons. In one of the first reports of this kind, Moore and colleagues showed that in rat hippocampal pyramidal cells, alcohol significantly reduced the M-current and blocked typical somatostatin-induced augmentation of the M-current (Moore et al. 1990). They also demonstrated that this effect of alcohol was mediated through mechanisms distinct from the muscarinic receptor, as bath application of atropine did not alter the alcohol-induced suppression of M-channel activity (Moore et al. 1990). More recent data in dissociated rat VTA dopaminergic neurons confirms that alcohol reduces M-current by acting directly on K_V7 channels in a voltage-independent manner, suggesting that the site of action of alcohol is distinct from the voltage-sensing regions of K_V7 channel subunits (Koyama et al. 2007). Additionally, this effect is conserved across species, as the M-channel ortholog in *Drosophila*, *dKCNQ*, is a direct target of alcohol (Cavaliere et al. 2012, 2013).

While the majority of studies in this area have focused on the acute effects of alcohol on M-channel function, Kang and colleagues have recently examined the effects of more chronic alcohol administration on M-channel function. They demonstrate that repeated alcohol exposure results in increased excitability of neurons in the LHb, as evidenced by increased evoked spike firing, as well as a reduction in the mAHP and decreased ability of XE-991 to increase LHb cell firing (Kang et al. 2017). The authors argued that these effects of alcohol on neuron physiology are attributable to a loss of M-channel activity because of an overall reduction of both $K_V7.2$ and $K_V7.3$ subunit expression in LHb neurons (Kang et al. 2017; Shah et al.

2017). Similarly, $K_V7.2$ subunits in the NAc are differentially trafficked between detergent soluble and insoluble membrane fractions after chronic alcohol consumption, possibly due to changes in $K_V7.2$ SUMOylation that could alter M-channel function (McGuier et al. 2016). These changes in cellular expression profiles and function of M-channels following alcohol exposure (Fig. 2) provide support for using K_V7 channel positive modulators to reduce alcohol consumption associated with AUD.

7.3 Retigabine and Drinking

A number of preclinical studies demonstrate that increasing M-channel activity can alter alcohol-associated behaviors and, importantly, may represent a promising pharmacological target for treating AUD. Retigabine, a K_V7 channel opener for treating epilepsy, has shown great promise in preclinical models as one such potential treatment. Initial demonstrations show that systemic retigabine administered acutely reduces alcohol consumption in rats in both a limited-access model of alcohol consumption (Knapp et al. 2014) and a more chronic alcohol-drinking model (McGuier et al. 2016). Interestingly, McGuier and colleagues demonstrated that retigabine was more effective in "high-drinking" rats than "low-drinking" rats and that positive modulation of K_V7 in the NAc was similarly effective in reducing alcohol consumption in "high-drinking" rats (McGuier et al. 2016). In a mouse model of chronic alcohol consumption, retigabine was highly effective at reducing consumption in mice showing a high-drinking phenotype (Rinker et al. 2017). Additionally, repeated, prophylactic administration of retigabine decreased hippocampal sensitivity to an acute intravenous injection of alcohol, i.e., retigabine decreased alcohol-induced changes in hippocampal EEG activity in rabbits (Zwierzynska et al. 2015). In a subsequent study, Zwierzynska and colleagues demonstrated that chronic retigabine administration blocked alcohol-induced changes in EEG activity both during forced alcohol administration and during abstinence, highlighting the potential of retigabine to prevent alcohol dependence-related functional changes in activity of the frontal cortex, hippocampus, and midbrain (Zwierzynska et al. 2016). As well, LHb M-channels are sensitive to repeated alcohol exposure, and Kang and colleagues determined that microinfusion of retigabine into the LHb, but not the nearby paraventricular nucleus of the thalamus or the mediodorsal thalamic nuclei, significantly reduced alcohol consumption and alcohol withdrawal-induced anxiety (Kang et al. 2017). Taken together, these preclinical studies demonstrate that retigabine, or other K_V7 channel openers, holds great therapeutic potential for treating AUD. Despite these promising results, only one study to date has examined the effects of retigabine in a clinical population of moderate social drinkers to determine interactive effects of alcohol on retigabine pharmacodynamics and pharmacokinetics (Crean and Tompson 2013). Thus, future studies are essential to determine the efficacy of K_V7 channel positive modulators in treating AUD.

8 GIRK Channels and Alcohol

8.1 Genetics

A genome-wide QTL mapping study identified *Kcnj9* (a gene encoding the GIRK3 subunit) as one of the genetic determinants of alcoholism in mice (Buck et al. 2012). This gene was also associated with withdrawal from alcohol and other sedative hypnotics (Herman et al. 2015; Kozell et al. 2009), alcohol drinking (Tarantino et al. 1998), alcohol-conditioned aversion (Risinger and Cunningham 1998), and acute sensitivity to alcohol (Crabbe et al. 1994; Demarest et al. 1999). In BXD RI strains, *Kcnj3* (a gene encoding the GIRK1 subunit) transcript levels in the NAc positively correlated with voluntary alcohol drinking in nondependent mice, while *Kcnj6* (a gene encoding the GIRK2 subunit) in the PFC negatively correlated with drinking in nondependent animals (Rinker et al. 2017). Following CIE exposure, significant adaptations in *Kcnj3* and *Kcnj6* transcript levels in the NAc have been detected (Rinker et al. 2017). In addition, there was a positive correlation between *Kcnj9* and the change in voluntary drinking induced by alcohol dependence (Rinker et al. 2017). The genetic evidence in mice was correlated with behavioral studies in GIRK-knockout models. For example, GIRK2$^{-/-}$ mice showed reduced conditioned taste aversion and failed to develop a conditioned place preference for alcohol (Hill et al. 2003). Additionally, mice lacking GIRK3 exhibited excessive alcohol drinking (Herman et al. 2015) and demonstrated less severe withdrawal symptoms compared to their wild-type littermates (Kozell et al. 2009).

The involvement of *KCNJ* genes in the development of alcohol use disorders has also been identified in humans. One SNP, rs2836016 in *KCNJ6*, was found to be associated with alcohol dependence in adults. In addition, this *KCNJ6* SNP was significantly associated with hazardous drinking, as defined by the Alcohol Use Disorders Identification Test (AUDIT), in adolescents but only in those exposed to early life stress (Clarke et al. 2011). Another study reported several SNPs in the promoter region of *KCNJ6* in AUD subjects and in offspring at high risk to develop an AUD (Kang et al. 2012). In this study, electroencephalogram was used to record the theta event-related oscillations (EROs) that reflect processes underlying frontal inhibitory control, conscious awareness, and memory and processes that are often impaired in individuals with drug and alcohol use disorders. The results demonstrated a correlation between *KCNJ6* SNPs and theta oscillations across the scalp, with the strongest associations for the frontal phenotype (Kang et al. 2012). A significant association of *KCNJ6* SNP for nicotine dependence has also been reported (Saccone et al. 2007). Together, behavioral and genetic evidence in rodents and humans suggest the involvement of *KCNJ* family of genes with alcohol consumption and behaviors related to heavy drinking.

8.2 Alcohol and GIRK Channel Function

It is largely accepted that alcohol affects multiple neurotransmitters in the brain, many of which (i.e., dopamine and GABA) are linked to GIRK channel activation.

For example, alcohol enhances $GABA_B$-mediated inhibitory postsynaptic transmission on rat VTA neurons by facilitating GIRK currents (Federici et al. 2009). Withdrawal from repeated in vivo alcohol exposure produced a profound decrease in D2-/GIRK-mediated inhibition in VTA neurons of mouse brain slice while having no effect on $GABA_B$-/GIRK-mediated inhibition (Perra et al. 2011). Recent findings from our group demonstrated that monoamines, including dopamine, serotonin, and norepinephrine, decreased evoked firing of lOFC neurons in C57BL/6J mice via the activation of G_i-coupled D2, 5HT1A, and α2-adrenergic receptors, respectively (Nimitvilai et al. 2017b). This effect was GIRK-dependent as blocking GIRK channel with barium attenuated monoamine inhibition, and the GIRK1-subunit selective activator, ML297, by itself reduced evoked spiking. Following CIE exposure, however, the inhibitory effects of each monoamine or ML297 were blunted (Nimitvilai et al. 2017b), indicating the importance of monoamine/GIRK system as a modulator of lOFC excitability and suggesting that disruption of this process could contribute to various deficits associated with alcohol use disorder. As mentioned earlier, alcohol at intoxicating concentrations in humans can directly activate GIRK channels independently from GPCR signaling pathway. In *Xenopus* oocytes co-injected with mRNAs encoding GIRK1/GIRK2 subunits or GIRK1/GIRK4 subunits, alcohol (100–200 mM) induced strong K^+ currents, and this effect was blocked by barium, suggesting a direct action of alcohol on GIRK channels (Kobayashi et al. 1999). A similar finding was reported for GIRK-mediated currents in cerebellar granule cells (Lewohl et al. 1999). Recently, a high-resolution crystal structure of a GIRK channel (Aryal et al. 2009) combined with an alcohol-tagging approach (Bodhinathan and Slesinger 2013) revealed that GIRK channels contain an alcohol binding pocket located at the interface between two adjoining subunits within the cytoplasmic domains. The presence of alcohol in the pocket induces conformational changes and increases the affinity of PIP2 that helps stabilize the open state of the GIRK channel. Mutation of a leucine (L257) within the alcohol binding pocket significantly decreases alcohol-induced GIRK currents (Aryal et al. 2009). Therefore, changes in physical and chemical nature of the alcohol binding pocket could interfere with alcohol action on GIRK channels.

8.3 GIRK Channel Ligands and Drinking

Imbalance or dysfunction of GIRK channels has been implicated in altering neuronal excitability and is linked to many neuropsychiatric diseases and SUDs. As mentioned above, a number of studies demonstrate that GIRK channels can be activated directly by acute alcohol (Bodhinathan and Slesinger 2013; Kobayashi et al. 1999; Lewohl et al. 1999) and that GIRK function is blunted following chronic alcohol exposure (Nimitvilai et al. 2017b). In addition, the action of many G_i-coupled neurotransmitters, including dopamine, serotonin, and GABA, that are linked to GIRK channel activation is modulated by alcohol (Nimitvilai et al. 2017b; Federici et al. 2009; Perra et al. 2011). Genetic studies also depict the association of *Kcnj* genes encoding GIRK channels in alcohol use disorder and dependence (Rinker and

Mulholland 2017; Rinker et al. 2017; Buck et al. 2012; Clarke et al. 2011). Therefore, selective manipulation of GIRK channels may represent a promising target for treating excessive drinking and relapse.

In 2012, baclofen, a $GABA_B$ agonist that is linked to GIRK channel activation, was approved as a treatment for alcohol addiction in France and is currently under clinical trials in the USA. It shows promising effects in managing alcohol withdrawal symptoms, reducing alcohol craving and consumption, and promoting alcohol abstinence in alcoholic animal models and humans, without serious or severe side effects (Addolorato et al. 2012; Maccioni and Colombo 2009; Morley et al. 2014). An increase in sedation on the BAES scale was reported in human subjects; however, no clinically significant sedative side effects, including sedation, tiredness, and sleepiness, were reported during the treatment session, confirming the safety of baclofen in alcohol-dependent patients (Evans and Bisaga 2009; Leggio et al. 2012). Despite these positive findings, other studies reported a lack of efficacy of baclofen in some alcohol-dependent individuals (Garbutt et al. 2010; Leggio et al. 2010) and a severe sedative effect of the drug (Garbutt et al. 2010). One clinical trial study examined the possible role of DRD4, a genetic modulator associated with risk of alcohol dependence (AD), on the effect of baclofen (Leggio et al. 2013). There is evidence of a robust relationship between urge for alcohol and the DRD4 or 5-HTTLPR polymorphisms, i.e., the presence and absence of the DRD4 allele 7-repeat or the short and long allele in the 5-HTTLPR promoter region (Kenna et al. 2012; McGeary 2009). When baclofen was given to AD participants, less drinking was observed in subjects with $\geq$7 DRD4 repeats, while the opposite was observed in AD patients with $<$7 DRD4 repeats. Baclofen caused a reduction in alcohol consumption regardless of 5HTTLPR form; however, individuals with a homozygous LL genotype drank significantly less than those with SS/SL genotype (Leggio et al. 2013). Therefore, the ability of baclofen to reduce alcohol consumption might be limited to specific endophenotypes of AD individuals. At present, there is no treatment for alcohol dependence that directly targets GIRK channels. One compound, ML297, has been recently designed to directly activate GIRK1-containing GIRK channels (Kaufmann et al. 2013) and has been shown to decrease anxiety-related behavior and exhibit antiepileptic properties without affecting locomotor activity or conditioned place preference (Kaufmann et al. 2013; Wydeven et al. 2014). It will be interesting to examine whether ML297 can also be used to alleviate symptoms related to alcohol withdrawal that are thought to contribute to the risk of relapse. Although both ML297 and alcohol enhance GIRK channel activity, ML297 may help restore normal GIRK channel tone that is lost following chronic alcohol exposure and reduce craving for alcohol.

9 Conclusions and Future Directions

In this chapter, we have summarized the effects of chronic alcohol exposure on intrinsic excitability and adaptations in expression and function of K_{Ca}, K_v7, and GIRK channels across different alcohol exposure models and species (*Drosophila*,

rodent, monkey, and humans). In general, transcript levels of genes in these K^+ channel families negatively correlate with higher levels of alcohol intake. Evidence presented here suggests that downregulation of $K_{Ca}2$ and K_V7 channel function underpins the reduction in AHP amplitude and increased evoked cell firing after chronic alcohol exposure. In many cases, positive modulators of these channels restore or prevent aberrant physiology and behaviors that result from prolonged alcohol exposure, including alcohol-seeking and withdrawal- and anxiety-related behaviors (Table 4). Indeed, $K_{Ca}2$ and K_V7 channel positive modulators reduce alcohol intake across many preclinical models of drinking and seeking, and both channels play a critical role in heavy alcohol drinking in dependent and nondependent rodents (Padula et al. 2013, 2015; Rinker et al. 2017; McGuier et al. 2016). Together, these findings reveal genetic variations and adaptations in $K_{Ca}2$ and K_V7 channels that are important for the plasticity of intrinsic excitability and a heavy drinking phenotype.

The goal of preclinical studies is to identify neural mechanisms that underpin aberrant drug-seeking and relapse-like behaviors. As presented in this chapter, $K_{Ca}2$, K_V7, and GIRK channels emerged from these preclinical studies as promising "translational" therapeutic targets for treating alcohol use disorder. The next step in the progression to FDA approval is to determine the efficacy of compounds that target $K_{Ca}2$, K_V7, and GIRK channels in clinical trials of treatment-seeking individuals diagnosed with alcohol use disorder. Because activators of these channels have anticonvulsive properties, sedation and cognitive impairments may be an unwanted side effect. A small-scale trial in social and moderate drinkers (ClinicalTrials.gov record number: NCT01342341) was recently completed with chlorzoxazone, an FDA-approved drug prescribed as a skeletal muscle relaxant (Chou et al. 2004) that acts as a $K_{Ca}2$ channel positive modulator (Cao et al. 2001). Although chlorzoxazone was well tolerated in this moderate social drinking population, the dosing schedule used in this trial did not reduce the number of alcohol drinks across 2 weeks of treatment. These findings are in stark contrast to the preclinical studies showing that $K_{Ca}2$ channel positive modulators decrease consumption. The reasons for the discrepancy between the preclinical studies and this clinical trial are unclear but may relate to the short half-life, low EC_{50} for $K_{Ca}2$ channels, off-target actions of chlorzoxazone, conservative dosing approach, or population of drinkers that were recruited for the trial.

Similar to $K_{Ca}2$ channels, an FDA-approved drug, retigabine, acts as a positive modulator of K_V7 channels and is used to treat partial-onset seizures, and acute doses are well tolerated in moderate social drinkers (Crean and Tompson 2013). However, extended retigabine use produces pigment changes in the retina and skin due to accumulation of retigabine dimers with low solubility. Although the pigment changes are reversible, GlaxoSmithKline withdrew retigabine from the market in 2017. While these findings are somewhat discouraging, there are analogs of retigabine with selectivity for brain-specific K_V7 channel subtypes with chemical scaffolds that appear not to form insoluble dimers. Thus, despite some setbacks, there is continued enthusiasm for developing additional $K_{Ca}2$ and K_V7 channel positive modulators as pharmacotherapeutics for treating alcohol use disorder,

Table 4 Summary of effects of $K_{Ca}2$ and K_V7 channel ligands on alcohol-related behaviors

Alcohol-related behavior	Model	Species	Drug	Route of administration	Ligand outcome	Reference
$K_{Ca}2$ channels						
Alcohol-seeking	Alcohol self-administration	Rat	1-EBIO	NAc microinfusion	↓ Breakpoint for responding	Hopf et al. (2010)
Alcohol withdrawal	Chronic intermittent alcohol	Mouse	1-EBIO	IP	↓ HICs	Mulholland et al. (2011)
Voluntary drinking	IAA	Rat	Chlorzoxazone	IP	↓ Intake	Hopf et al. (2011)
Voluntary drinking	IAA	Mouse	CyPPA	IP	↓ Intake	Padula et al. (2013)
Voluntary drinking	2-bottle, limited-access drinking	Mouse	Apamin	NAc microinfusion	↑ Intake	Padula et al. (2015)
Extinction learning	Extinction and reinstatement of alcohol-seeking	Rat	Apamin	IP and IL-mPFC microinfusion	↑ Extinction learning and ↓ spontaneous recovery	Cannady et al. (2017)
K_V7 channels						
Acute intoxication	Clinical laboratory study	Human	Retigabine	PO	N/C in alcohol-induced deficits	Crean and Tompson (2013)
Voluntary drinking	IAA	Rat	Retigabine	IP and NAc microinfusion	↓ Intake	McGuier et al. (2016)
Alcohol-seeking	2-bottle, limited-access drinking	Rat	Retigabine	IP	↓ Intake	Knapp et al. (2014)
Alcohol dependence	Forced alcohol access	Rabbit	Retigabine	PO	↓ Alcohol-induced changes in EEGs	Zwierzynska et al. (2016)
Convulsions	IAA	Rat	XE-991	IP	↑ Spontaneous seizure-like activity	McGuier et al. (2016)
Voluntary drinking	IAA	Mouse	Retigabine	IP	↓ Intake	Rinker et al. (2017)

Anxiety-like behaviors	Repeated IP alcohol	Rat	Retigabine	LHb microinfusion	↓ Anxiety-like behaviors	Kang et al. (2017)
Voluntary drinking	IAA	Rat	Retigabine	LHb microinfusion	↓ Intake	Kang et al. (2017)
Voluntary drinking	IAA	Rat	Retigabine	MDT and PVN microinfusion	N/C in intake	Kang et al. (2017)

HICs handling-induced convulsions, *IL-mPFC* infralimbic medial prefrontal cortex, *IP* intraperitoneal, *IAA* intermittent access to alcohol, *EEG* electroencephalogram, *MDT* mediodorsal thalamic nucleus, *PO* per os, *PVN* paraventricular nucleus of the thalamus

especially in light of the vast preclinical genetic, functional, and pharmacological evidence supporting a role for these channels in alcohol-seeking behaviors. Finally, based on the compelling preclinical and human genetic evidence, future clinical studies are necessary to validate *KCNQ* SNPs as pharmacogenetic targets for a precision medicine approach for treating alcohol addiction.

In addition, although this chapter was focused on the role of discrete K^+ channel subtypes in alcohol addiction, emerging evidence reveals that plasticity of intrinsic excitability contributes to addiction of all abused substances (Kourrich et al. 2015; Kourrich and Thomas 2009) and neuropsychiatric disorders (Beck and Yaari 2008). For example, protracted withdrawal from chronic morphine exposure increased the intrinsic excitability of NAc shell medium spiny neurons in rats (Wu et al. 2013), and this was accompanied by an attenuation of the apamin-sensitive AHP current in the morphine-withdrawn rats. Interestingly, members of the *KCNN* family of genes have links with opioid and alcohol addiction (Padula et al. 2015), and multiple studies have implicated *KCNN3* mutations in schizophrenia (Bowen et al. 2001; Cardno et al. 1999; Chandy et al. 1998). A rare frameshift mutation in *KCNN3* that generates a dominant-negative form of this channel was reported in a patient with schizophrenia (Bowen et al. 2001), and longer polyglutamine repeat alleles in $K_{Ca}2.3$ channels associate with negative symptoms and cognitive performance in schizophrenics (Cardno et al. 1999; Grube et al. 2011). Importantly, the rare frameshift mutation and longer polyglutamine repeats in *KCNN3* suppress $K_{Ca}2$ channel surface expression and function (Grube et al. 2011; Miller et al. 2001). These findings are consistent with the overlap of some genes with abused substances and psychiatric diseases (Agrawal et al. 2012) and suggest that neural mechanisms that underlie adaptations in intrinsic excitability are critical factors that drive the risk for and emergence of neuropsychiatric disorders.

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Part III

Neuroimmune System

Hepatic Immune System: Adaptations to Alcohol

Adam Kim, Rebecca L. McCullough, Kyle L. Poulsen, Carlos Sanz-Garcia, Megan Sheehan, Abram B. Stavitsky, and Laura E. Nagy

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Adam Kim, Rebecca L. McCullough, Kyle L. Poulsen, Carlos Sanz-Garcia, and Megan Sheehan equally contributed to this work.

Abram B. Stavitsky posthumous contribution.

A. Kim · K. L. Poulsen · C. Sanz-Garcia
Department of Pathobiology, Center for Liver Disease Research, Cleveland Clinic, Cleveland, OH, USA

R. L. McCullough
Department of Pathobiology, Center for Liver Disease Research, Cleveland Clinic, Cleveland, OH, USA

Department of Molecular Medicine, Case Western Reserve University, Cleveland, OH, USA

M. Sheehan
Department of Molecular Medicine, Case Western Reserve University, Cleveland, OH, USA

A. B. Stavitsky
Department of Pathobiology, Center for Liver Disease Research, Cleveland Clinic, Cleveland, OH, USA

Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH, USA

L. E. Nagy (✉)
Department of Pathobiology, Center for Liver Disease Research, Cleveland Clinic, Cleveland, OH, USA

Department of Molecular Medicine, Case Western Reserve University, Cleveland, OH, USA

Department of Gastroenterology, Center for Liver Disease Research, Cleveland Clinic, Cleveland, OH, USA

Cleveland Clinic, Lerner Research Institute, Cleveland, OH, USA
e-mail: nagyL3@ccf.org

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_88

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Abstract

Both the innate and adaptive immune systems are critical for the maintenance of healthy liver function. Immune activity maintains the tolerogenic capacity of the liver, modulates hepatocellular response to various stresses, and orchestrates appropriate cellular repair and turnover. However, in response to heavy, chronic alcohol exposure, the finely tuned balance of pro- and anti-inflammatory functions in the liver is disrupted, leading to a state of chronic inflammation in the liver. Over time, this non-resolving inflammatory response contributes to the progression of alcoholic liver disease (ALD). Here we review the contributions of the cellular components of the immune system to the progression of ALD, as well as the pathophysiological roles for soluble and circulating mediators of immunity, including cytokines, chemokines, complement, and extracellular vesicles, in ALD. Finally, we compare the role of the innate immune response in health and disease in the liver to our growing understanding of the role of neuroimmunity in the development and maintenance of a healthy central nervous system, as well as the progression of neuroinflammation.

Keywords

Alcoholic liver disease · Cytokines · Hepatic macrophages · Innate immunity

1 Overview of Alcoholic Liver Disease

Alcoholic liver disease (ALD) is a complex condition caused by chronic alcohol abuse. It accounts for 40% of deaths from liver disease in the United States and Europe alone (Hilscher and Shah 2016) and affects thousands of patients each year. While entirely preventable, liver-related mortality from alcohol abuse remains a global burden, even as awareness of the risks increases. ALD makes up 4% of total mortality and 5% of disability-adjusted life years globally (Singal and Anand 2013). ALD manifestation is not purely dose-dependent, but varies based on a number of identified risk factors. This includes the duration and pattern of alcohol consumption, as well as individualized genetic and environmental factors (O'Shea et al. 2010).

The spectrum of ALD symptoms includes steatosis, fibrosis, alcoholic hepatitis, and cirrhosis, which can occur sequentially or simultaneously in individual patients. Steatosis, or fatty liver, develops after short-term consumption of alcohol and is primarily asymptomatic (Singal et al. 2016). Liver fibrosis and cirrhosis, which are characterized by an increase in liver collagen and impaired liver function, are more

severe forms of injury. In particular, alcoholic hepatitis (AH), which can be present at any point on the spectrum, has a high rate of short-term mortality. Twenty-eight-day mortality rates for severe AH patients are 40–50% (Singal et al. 2016). While abstinence improves clinical outcomes after diagnosis, cirrhosis is irreversible and associated with negative patient outcomes.

One of the major challenges associated with ALD is the difficulty to diagnose patients at an early point in disease progression. Often, clinical diagnosis comes with very advanced stages of the disease, which significantly limits treatment options (Yeluru et al. 2016). Liver transplants are the last step in treatment for ALD patients, but social stigma and concern involving potential relapse have prevented this from becoming common, particularly in the United States. There is a need for more effective screening and treatment options for patients with ALD.

The stress-innate immunity-homeostasis axis (S-I-HA) paradigm posits that over eons the selective pressures of infectious, traumatic, and chemical-toxic stresses induced the evolution of innate immune pathways which respond promptly to these stresses by up- and/or down-modulating homeostasis and thus influencing fitness and survival (Stavitsky 2007). While the evolution of the immune system was initially linked to selective microbial pressure, later, it was proposed that innate immunity evolved in response to "danger signals" from tissues injured by pathogens, trauma, and toxins (Matzinger 2002). Both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are critical signals to activate innate immunity in response to infectious, traumatic, and toxic stresses, such as ethanol. PAMPs and DAMPs selectively induce networks of interactive, reparative, and pathogenic innate immune pathways which modulate homeostasis (Stavitsky 2007).

The pathophysiological mechanisms for the development of ALD are complex and can be considered as an impaired ability of the innate immune system to elicit an appropriate wound healing response. Importantly, chronic alcohol consumption increases exposure to both PAMPs and DAMPs in the liver. Ethanol metabolism leads to an accumulation of reactive oxygen species (ROS), which causes oxidative stress and endoplasmic reticulum stress in hepatocytes (Louvet and Mathurin 2015). These cytotoxic effects lead to hepatocyte injury and death, causing the release of DAMPs. While the specific array of DAMPs released upon ethanol exposure is not well understood, DAMPs can include the ficolins, heat shock proteins (HSPs), high mobility box 1 (HMGB1), S100 proteins, advanced glycation end products (AGE), and chromatin (Fleshner and Crane 2017). Alcohol also impairs the barrier function of the intestine, increasing the concentration of LPS and other PAMPs in the portal circulation (Chen et al. 2016a). Increased concentrations of PAMPs/DAMPs trigger the activation of macrophages and neutrophils. Importantly, chronic ethanol also enhances PAMP/DAMP signaling, thus further exacerbating the activation of the immune response and inappropriately increasing the production of pro-inflammatory cytokines and chemokines (Wang et al. 2012). AH, in particular, is characterized by an inappropriate and non-resolving inflammatory response, leading to the development of excessive and dangerous inflammation that plays a key role in pathogenesis of the disease (Colmenero et al. 2007).

The innate and adaptive immune responses function autonomously as well as collaboratively to normalize or dysregulate homeostasis (Sharland and Gorrell 2009). The innate immune pathways activate and/or differentiate many cell types to produce a rich variety of circulating and membrane-bound molecules required for innate and adaptive immune responses (Fearon and Locksley 1996). Here we will review the basics of innate and adaptive immune responses in the liver, as well as the adaptations/dysregulation of these responses in ALD. Hepatic immune responses to ethanol will be contrasted to more recent studies characterizing the importance of the effects of ethanol on activation of innate immune functions in the brain.

2 The Hepatic Immune System

The liver is an extremely tolerogenic organ. Portal blood from the intestine, rich in both bacterial and food antigens, continuously challenge the liver to maintain balance between self and non-self. Alcohol impacts both the innate and adaptive immune response in the liver, resulting in a loss of tolerance, thus increasing the potential for persistent inflammation (Nagy 2015).

2.1 Innate Immunity

Diverse innate immune pathways and networks deploy many cell types and molecules in the liver to maintain homeostasis and promote wound healing and tissue repair in the face of toxins, such as alcohol. The liver is enriched in resident innate immune cells including Kupffer cells, dendritic cells (DCs), natural killer (NK) cells, and NKT cells (Fig. 1). Injury also results in the recruitment of peripheral immune cells including neutrophils and infiltrating monocytes. Chronic, heavy alcohol exposure results in the dysregulation of the innate immune activity of these cells, contributing to the progression of the non-resolving inflammation characteristic of AH and ALD (Nagy 2015; Gao et al. 2011).

2.1.1 Kupffer Cells

Kupffer cells are macrophages resident in the liver sinusoids and are important contributors to the progression of ALD. They are among the first cells exposed to alcohol-induced, microbe-derived PAMPs originating from the gut, including LPS and peptidoglycans. Increased intestinal translocation of bacterial LPS during alcohol consumption is central to inducing TLR4-mediated Kupffer cell activation (Nakamoto and Kanai 2014; Roh and Seki 2013; Wheeler et al. 2001). In addition, chronic alcohol intake also sensitizes Kupffer cell responses to LPS-mediated activation (Wang et al. 2012). Activated Kupffer cells produce inflammatory mediators (e.g., TNFα and ROS) that contribute to hepatocyte necrosis and apoptosis and generation of extracellular matrix proteins leading to alcoholic liver injury and fibrosis (Liu et al. 2017; Xu et al. 2017).

Necrotic hepatocytes release DAMPs, exosomes, and microRNAs that also can activate Kupffer cells in a sterile inflammatory manner aggravating the progression

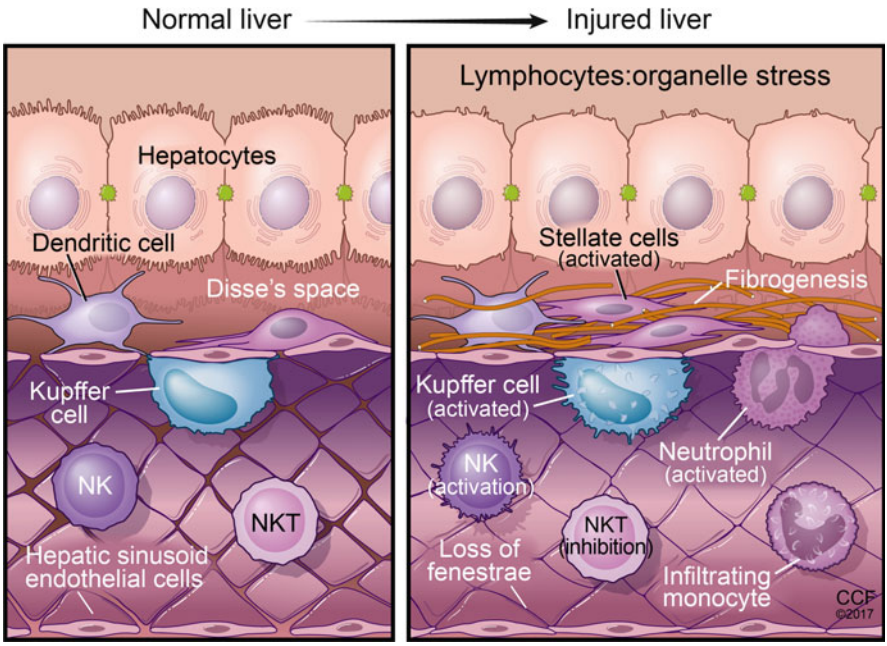

Fig. 1 Resident immune cells with the specialized architecture of the liver. Kupffer cells, the resident macrophage in the liver, are localized in the hepatic sinusoids, in close proximity to liver sinusoidal endothelial cells and hepatocytes. Other resident immune cells, including NK and NKT cells, are also located within the sinusoids, while hepatic stellate cells, responsible for generation of extracellular matrix during fibrosis, are localized in the space of Disse. Infiltrating monocytes and neutrophils are also recruited to the liver in response to injury

of the disease (Fleshner and Crane 2017). Recent research has focused on the identification of anti-inflammatory molecules that might be effective a breaking the cycle of continued dysregulation of Kupffer cell activation, identifying several specific pathways that may be effective at normalizing Kupffer cell signal transduction, including adiponectin (An et al. 2012; Gao 2012), IL-10 (Mandal et al. 2010) and hyaluronic acid of an average molecular weight of 35 kDa (HA35), which acts via interactions with CD44-dependent signaling (Saikia et al. 2017).

Resident macrophages exhibit a tremendous phenotypic plasticity, dependent on the local metabolic and immune environment. Macrophages are sufficiently plastic to integrate multiple signals, such as those from microbes, damaged tissues, and the normal tissue environment (Murray 2017). The balance of the polarization of Kupffer cells is very important for the tight regulation of the development of liver injury, with phenotypes ranging from pro-inflammatory, anti-inflammatory, as well as phenotypes promoting the resolution of fibrosis (Ju and Mandrekar 2015). Recent studies demonstrate that limiting the pro-inflammatory polarization of Kupffer cells could be a

protective strategy that prevents the progression of ALD. For example, IL-10, generated by anti-inflammatory macrophages, promotes selective pro-inflammatory macrophage cell death by apoptosis via paracrine activation of arginase (Wan et al. 2014).

2.1.2 NK Cells

NK cells play an important role in antiviral and antitumor defenses in the liver. However, NK cell function is suppressed in ALD [reviewed in (Gao et al. 2011)]. Thus, it is very unlikely that NK cells contribute to ethanol-induced hepatocellular damage. However, it is more likely that inhibition of NK cells by ethanol may play an important role in accelerating liver fibrosis. Many studies have focused on the crosstalk between hepatic stellate cells and NK cells in the progression of the fibrotic stages of ALD. For example, the oxidative stress resulting from chronic ethanol consumption induces increased levels of TGF-β and reduces IFN-γ signaling, blocking NK cell killing of activated HSC via TNF-related apoptosis-inducing ligand (TRAIL)-TRAIL receptor interactions (Ness et al. 2008). Inhibition of alcohol dehydrogenase 3 (ADH3) enhanced IFN-γ production, promoting the cytotoxic activity of NK cells against HSCs and protecting against liver fibrosis (Yi et al. 2014). Finally, IL-22, a member of the IL-10-like cytokine family, is also produced by NK cells. IL-22 activates STAT3 signaling pathway, increasing HSC senescence and reducing collagen deposition [reviewed in (Ceni et al. 2014)].

There remains a significant amount of controversy as to the role of NK cells in the progression of ALD. For example, the impact of acute versus chronic ethanol on NK cells may be different. In one study, acute alcohol ingestion decreased the number and cytotoxic function of NK cells; responses were normalized after 12–14 days but then increased after 8 weeks of continued alcohol ingestion (Ballas et al. 2012). Finally, it is not clear if ethanol impacts the differentiation of specific NK cell phenotypes, designated as memory-like or adaptive NK cells that develop after repeated stimulus, such as in response to cytomegalovirus infection in humans (Kovalenko et al. 2017).

2.1.3 NKT Cells

NKT cells, innate-like cells which are abundant in the liver sinusoids, are a heterogeneous group of T lymphocytes that recognize lipid antigens in the context of CD1d, a nonclassical MHC class I-like molecule. Although activation of NKT cells has been shown to induce hepatocellular damage in a variety of acute liver injury models, different subsets of NKT cells can play opposing roles in non-microbial liver inflammation (Gao et al. 2011). Type I NKT cells can be activated after injury, directly by recognition of cognate lipids or indirectly by TLRs ligands and cytokines, increasing the production of various cytokines such as IFN-γ and IL-4 and finally giving hepatocellular death neutrophils and macrophages infiltration [reviewed in (Kumar 2013; Cui et al. 2015; Mathews et al. 2016)]. Inhibition of type I NKT cells with exposure to retinoids protects against ALD (Maricic et al. 2015) and chronic alcohol consumption enhances cell maturation and activation of type I NKT cells (Zhang et al. 2015). However, Type II NKT cells have been shown to protect against ALD after sulfatide-mediated activation (Maricic et al. 2015) with a novel mechanism involving the release of all-trans retinoic acid to inhibit the functions of type I NKT cells.

Different subsets of NKT cells also differentially regulate fibrosis. For example, NKT cells can kill activated hepatic stellate cells and produce IFN-γ, which inhibits liver fibrosis (Park et al. 2009), whereas activation of NKT cells can also promote liver fibrosis via enhancing hepatocellular damage and promoting HSC activation (Jin et al. 2011; Syn et al. 2012). Therefore, the net impact of NKT cells on liver fibrosis is determined by the balance between these inhibitory and stimulatory effects of NKT cells. Identification of the precise impact of each NKT subset in liver disorders could potentially lead to the development of novel therapeutics.

2.1.4 Dendritic Cells (DC)

DCs are the most efficient antigen-presenting cells (APCs) of the immune system, playing a crucial role in innate and adaptive immune responses. Chronic alcohol ingestion can interfere with antigen presentation that is required to activate T and B cells and can impair dendritic cell differentiation (Ceni et al. 2014). In addition to acting as APCs, hepatic DCs also either aggravate or ameliorate hepatocellular damage via production of pro-inflammatory (Connolly et al. 2009) or anti-inflammatory cytokines (Bamboat et al. 2010) in various liver injury models. Alcohol consumption can modulate the functions of DCs (Heinz and Waltenbaugh 2007; Laso et al. 2007; Lau et al. 2006; Pascual et al. 2011) and subsequently impair the cellular response necessary for clearance of hepatitis virus (Szabo et al. 2010), likely contributing to the synergistic effect of alcohol and viral hepatitis on liver injury. However, it remains unknown whether DCs directly contribute the pathogenesis of alcoholic liver injury via increases in ROS production, TLR signaling, inflammasome activation, and Il-1β production (Pearce and Everts 2015).

2.2 Adaptive Immunity

In addition to the effects of ethanol on cells of the innate immune system, early studies identified both $CD8^+$ and $CD4^+$ T lymphocytes in liver biopsies from patients with AH and cirrhosis (Albano 2012; Chedid et al. 1993), implicating the adaptive immune system as a contributor to alcohol-induced hepatic inflammation.

Adaptive responses consist of cellular and humoral components, primarily mediated by B and T lymphocytes. $CD4^+$ T cells play a critical role in the activation and differentiation of both innate and adaptive immune cells, including macrophages, $CD8^+$ T cells, and B cells, while $CD8^+$ T cells aid in the clearance of infected and cancerous cells (Pasala et al. 2015). B cells produce antibodies against both ingested and self-antigens, important for the elimination of pathogenic material. In fact, immunogenic molecules have been reported in ALD, including liver-specific autoantigens against alcohol-metabolizing enzymes (McFarlane 2000; Sutti et al. 2014) and advanced lipid peroxidation products (Albano 2012; Mottaran et al. 2002).

Chronic, heavy alcohol consumption differentially effects tissue and systemic immune responses. Therefore, while chronic alcohol increases inflammatory responses in tissues, such as the liver and gut, individuals with alcohol use disorder (AUD) are prone to increased incidence of bacterial and viral infections (Pasala et al. 2015; Massey et al. 2015) and are considered to be "immunocompromised hosts." Chronic alcohol abuse, as

well as binge drinking, causes lymphopenia (reduced peripheral T cell numbers) (Liu 1973; McFarland and Libre 1963; Tonnesen et al. 1990) and disrupts the balance between T cell subtypes. Reduction of naive $CD4^+$ and $CD8^+$ cells with concurrent increases in memory T cells have been reported in several studies (Cook et al. 1994, 1995; Song et al. 2002; Zhang and Meadows 2005). Chronic alcohol also lowers the numbers of circulating B cells, in particular conventional memory B cells (involved in responding to repeated exposure of a priming antigen) (Cook et al. 1996), likely contributing to inadequate responses of patients with ALD to new antigens (Pasala et al. 2015).

Two distinct populations of effector T cell (T_{eff}) subtypes, Th17 and regulatory T cells (T_{reg}), are pivotal regulators of immune homeostasis (Abe et al. 2013), with the reciprocal relationship between Th17 and T_{reg} dictating hepatic tolerance. T cell differentiation is mediated by the local cytokine environment; alcohol's effect on the innate immune response stimulates the production of a multitude of cytokines, including IL-6. Together with TGF-β and IL-21 [in mice (Veldhoen et al. 2006)] or IL-23 [in humans (Wilson et al. 2007)], IL-6 promotes differentiation of naïve $CD4^+$ T cells to Th17, effectively driving T_{reg} depletion. Th17 cells secrete chemokines (e.g., IL-17A) that recruit and activate neutrophils and macrophages to induce inflammation. Patients with AH and cirrhosis have higher plasma IL-17, as well as IL-17^+ hepatic inflammatory foci (Lemmers et al. 2009). The numbers of IL-17^+ cells correlates with disease severity in ALD patients.

3 Circulating Mediators of Inflammation in ALD: Cytokines, Chemokines, Complement, and Extracellular Vesicles

3.1 Chronic Ethanol Sensitizes Kupffer Cells to Activation by PAMPs and DAMPs

Kupffer cells, the resident hepatic macrophage, display a tremendous phenotypic plasticity. The activation state of Kupffer cells in the healthy liver promotes hepatic tolerance. In ALD, Kupffer cells exhibit a shift in macrophage polarization, with an increase in the pro-inflammatory M1 phenotype and a decrease in the anti-inflammatory/tissue repair phenotype (M2) (Karakucuk et al. 1989). This shift in Kupffer cell phenotype increases their sensitivity to activation by PAMPs and DAMPs and contributes to the progression of disease (Fig. 2). Expression of pro-inflammatory signals, such as TNFα, IL-6, IL-8, and IL-18, increases, as well as production of reactive oxygen species (ROS). In chronic alcohol, Kupffer cell expression of TNFα contributes to the apoptosis and necrosis of liver hepatocytes (Hishinuma et al. 1990; McClain et al. 1998) and fibrosis caused by hepatic stellate cells (Karlmark et al. 2009). Activation of the inflammasome and generation of IL1-β is also an important contributor to the accelerating cycle of inflammation in the liver in response to chronic ethanol exposure (Petrasek et al. 2012, 2013).

The mechanisms for the sensitization of Kupffer cells to activation have been best studied in response to TLR4 ligands. Chronic ethanol feeding enhances TLR4 signaling via both the MyD88-dependent and -independent pathways (Wang et al. 2012). In the MyD88-

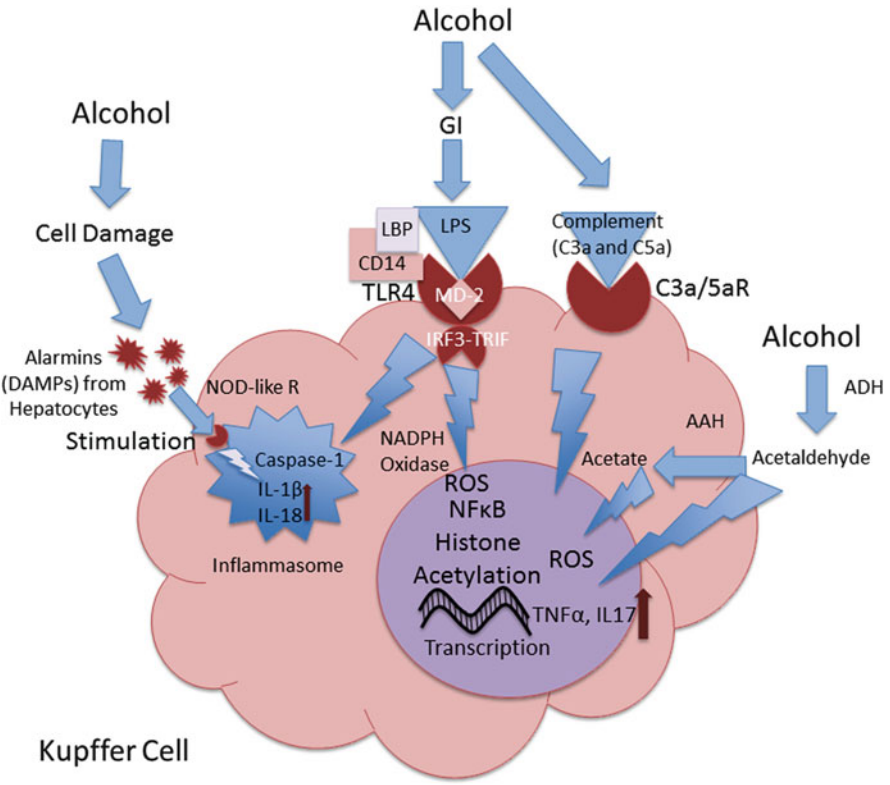

Fig. 2 Pathways of activation of Kupffer cells in response to ethanol. Kupffer cells, the resident macrophages in the liver, are activated by bacterial PAMPs from the gut, as well as DAMPs released from injured hepatocytes. Complement anaphylatoxins, C3a and C5a, as well as ethanol metabolism and production of reactive oxygen species, also contribute to activation of Kupffer cells during ethanol exposure. These activation pathways are integrated and result in dysregulated production of pro-inflammatory cytokines and chemokines. Reprinted with permission from Nagy (2015)

dependent arm of TLR4 signaling, TAK1 is activated, leading to NF-kB translocation to the nucleus and MAPK signaling (Akira and Takeda 2004); chronic ethanol increases activation of both NF-kB and MAPK family members, leading to increased transcription and mRNA stabilization of multiple cytokines and chemokine (Wang et al. 2012). TNFα also increases the expression of TLRs, thus increasing sensitivity of Kupffer cells to ROS and LPS in response to ethanol (Wang et al. 2012). The MyD88-independent pathway acts through TRIF1 to activate IRF3 and increase expression of type I interferons; this pathway is a critical regulator of the progression ALD (Petrasek et al. 2011). In particular, IRF3 expression in non-parenchymal cells contributes to upregulating pro-inflammatory cytokine expression in response to ethanol (Petrasek et al. 2011).

3.2 Chemokines and Recruitment of Immune Cells from the Periphery in ALD

Appropriate and effective immune cell trafficking is essential to host defense from pathogens. Whereas cytokines, interleukins, and complement play critical roles acting directly on tissues in response to a noxious stimuli like excessive alcohol consumption or toxicant exposure, chemokines (chemotactic cytokines) orchestrate the dynamics of cellular infiltration into sites of damage within tissues (Marra and Tacke 2014). Chemokines are a vast, redundant web of more than 50 ligands and 20 receptors (Marra and Tacke 2014). Chemokines can be secreted by many cell types in response to acute and chronic injury. Chemokines are classified into four different families based upon cysteine residues near their N-terminal, so-called CC, CXC, CX3C, and C (Charo and Ransohoff 2006). These chemokine families interact with G-protein-coupled receptors of a similar nomenclature, e.g., CCLs bind to CCRs and CXCLs bind CXCRs. The ligand-receptor interaction initiates intracellular events including Ca^{+2} mobilization, cytoskeletal rearrangement, and even cellular proliferation, that allow for immune cell transit into tissues (Marra and Tacke 2014). The complex choreography of immune cell recruitment in response to chemokines involves dynamic changes in chemokine gradients at the site of damage and egress of immune cells from the bone marrow.

3.2.1 Chronic Ethanol Enhances the Recruitment of Infiltrating Monocytes

Circulating monocytes are known to be key contributors to progression of ALD (Marra and Tacke 2014). In circulation, monocytes can be classified by expression of the surface receptor Ly6C, wherein Ly6C-high expressing monocytes are pro-inflammatory and Ly6C-low expressing monocytes are key to regression of injury and inflammation in animal models of fibrosis resolution (Ramachandran et al. 2012; Wang et al. 2014). These pro-inflammatory monocytes generate inflammatory cytokines in response to bacterial ligands like lipopolysaccharide or DAMPS released from damaged cells. In ALD, several chemokines are upregulated in the liver, including monocyte chemoattractant protein-1 (CCL2/MCP-1), CXCL1, CXCL2, CXCL5, CXCL8, and CXCL10 (Marra and Tacke 2014) as well as the pluripotent protein macrophage migration inhibitory factor (MIF) that contains a pseudo-E-L-R motif similar to the C-X-C chemokine family (Barnes et al. 2013; Marin et al. 2017). A seminal study identified a multifaceted role for CCL2 /MCP-1 in ALD progression. Mice deficient in CCL2 were protected from hepatocyte injury, steatosis, and excessive inflammatory cytokine production following ethanol feeding (Mandrekar et al. 2011). Interestingly, mice deficient in CCR2, the cognate receptor for CCL2, were not protected from ethanol-induced liver injury or inflammation, demonstrating the complex biology not yet fully understood with regard to chemokines and chronic disease, including ALD.

Infectious, traumatic, and toxic stresses induce the release of MIF from virtually all cell types by diverse innate immune mechanisms (Stavitsky 2007). In particular, hepatocytes are a critical source of MIF in response to chronic ethanol exposure (Marin et al. 2017). Studies have also provided strong evidence for the role of MIF in ALD

progression as an upstream regulator of chemokine synthesis might be an important aspect of chemokine-mediated contributions to chronic disease. Although MIF does have direct chemokine activity through interactions with CXCR2 and CXCR4, MIF can also direct chemokine synthesis (CCL2, CXCL10, and CXCL1) in the livers of ethanol-fed mice. MIF-deficient mice are protected from ethanol-induced liver injury and chemokine upregulation, as well as inflammatory monocyte accumulation and resident macrophage proliferation in the hepatic parenchyma (Barnes et al. 2013; Marin et al. 2017), possibly through a combination of MIF-mediated chemotaxis and MIF-dependent upregulation of chemokines in the liver.

3.2.2 Neutrophils in the Progression of ALD

Alcohol increases neutrophil recruitment to the liver; neutrophils are a very prominent feature of AH. While activated neutrophils release numerous harmful mediators such as H_2O_2, elastase, chloramine, and proteinase-3 (Neuman et al. 2015), recent studies revealed that patients with AH have better prognosis associated with neutrophil infiltration (Altamirano et al. 2014), suggesting a complex role for neutrophils in both injury and repair during the progression of ALD and AH. Recruitment of neutrophils to the liver is associated with increased expression of CXC subfamily members in patients with AH (Dominguez et al. 2009) and is dependent on TLR2 and 9 (Roh et al. 2015), as well as E-selectin (Bertola et al. 2013). Moreover, chemokines such as CXCL1, CXCL2, and CXCL5 were reduced in $TLR2^{-/-}$ and $TLR9^{-/-}$ mice suggesting the connection between those chemokines and neutrophil infiltration (Roh et al. 2015).

3.3 Complement

Complement, a component of the innate immune system that provides a link between the innate and adaptive immune response, is implicated in the immune system's response to ethanol. Complement primarily functions as a first line of defense against infection, acting to facilitate clearance of microbes and mediating inflammation by attracting macrophages and neutrophils. However, it acts as an aggressor in certain environments, including in immune and inflammatory diseases (Ricklin et al. 2016). At least two complement factors, C3 and C5, contribute to the pathogenesis of ALD; however, their exact role is not completely understood (Gao and Bataller 2011; Pritchard et al. 2007).

The complement system is activated via three separate pathways, which converge at the terminal component C3. The classical pathway is activated by C1q binding to an antibody-antigen complex. The lectin pathway, which converges with the classical pathway at C4, is activated upon the recognition of bacterial wall carbohydrates through mannose-binding lectins. The alternative pathway is initiated by the hydrolysis of C3 through a spontaneous process, also called tick-over, and provides an amplification loop that increases the overall complement response (Ricklin et al. 2016). Multiple complement pathways contribute to the phagocytosis of debris accumulated from dead or damaged cells including C1q, factor D, and the C5b-9 complex.

These pathways are activated and regulated by over 30 membrane-bound and circulating proteins and cognate receptors (Bohana-Kashtan et al. 2004). Regulatory proteins and receptors tightly control complement response to injury by modulating multiple steps of these activation pathways. C3 and C5 convertase assembly is prevented by a family of proteins, regulators of complement activation, which include CR1, CD46, and CD55 (Ricklin et al. 2016). Factor H controls tick-over activation and the amplification loop of the alternative pathway. CD59 prevents formation of the membrane attack complex, and clusterin and vitronectin also regulate this complex. Properdin, a plasma protein, acts as a positive modulator by stabilizing to the C3 convertase of the amplification loop. Expression of specific complement receptors, including C3aR and C5aR, is another important regulatory step that has been associated with crosstalk between the complement response and other immune systems (Ricklin et al. 2016). Other regulatory factors also play a role in complement activation, and an emerging theory suggests that these complex interactions may be context-specific (Ricklin et al. 2016).

Complement has complex pathophysiologic roles in that it has both damaging and beneficial effects, particularly in response to ALD. C1q contributes to inflammation and early liver injury (Cohen et al. 2010). However, complement is also involved in hepatic regeneration through transcriptional control after chemical injury (Min et al. 2016). Further, the alternative pathway may have a protective role after ethanol exposure by facilitating the clearance of apoptotic and necrotic cells in animal models (Cresci et al. 2015) (McCullough et al. 2018). Immunoreactive C5aR, a receptor of C5a, is increased in patients with AH (Shen et al. 2014). After ethanol-induced liver injury, mice lacking CD55, a complement regulator, showed worsened injury (Pritchard et al. 2007). The exact mechanisms of complement activation in patients with ALD are not well understood.

3.4 Extracellular Vesicles

Extracellular vesicles (EVs), including exosomes and microparticles, are a rapidly expanding field of study in many diseases (Bang and Thum 2012; Povero et al. 2014; Verma et al. 2016). Isolated EVs are enriched in exosomes (~20–100 nM particles) and microparticles (~80–200 nM particles). Much is still being discovered about exosomes in chronic diseases. How exosomal cargo is loaded, why exosome release is increased, and how exosomes home to certain tissues or deliver their cargoes are all active fields of study. The recent interest in EVs has transformed how we view interorgan and intercellular communication in ALD. The ability to rapidly isolate, identify cargo, and manipulate and administer exosomes to ALD patients will likely shape the future of therapeutic and diagnostic innovation in ALD.

EVs are increased in circulation in AH patients and following animal models of ethanol feeding (Verma et al. 2016; Momen-Heravi et al. 2015). EVs are potential diagnostic tools given the protein and nucleic acid cargoes they contain and protect which can change due to ongoing and/or progressing disease, like ALD. A study in AH patients identified the proteomic signature in serum-derived exosomes, and the CD40L contained within these vesicles was found to be released from injured hepatocytes and

was key in initiating macrophage activation in ethanol-fed mice (Verma et al. 2016). Exosomal microRNAs are potent mediators of inflammation and information transfer in models of ethanol feeding and fibrosis. One study identified that ethanol-treated hepatocytes, ethanol-fed mice, and ethanol-binged healthy human volunteers had increased exosome release. The vesicles from human volunteers, mice, and hepatocytes were all highly enriched in miR-122. The miR-122 contained in these exosomes sensitized monocytes to bacterial lipopolysaccharide and exacerbated cytokine release as is well-established in ALD and experimental models of ethanol exposure (Momen-Heravi et al. 2015).

4 Is the Hepatic Immune System a Target for Pharmacological Intervention?

Many of the signaling molecules and pathways known to be enhanced in ALD provide us with possibilities for developing pharmacological interventions or promising targets for the development of future novel therapies. Many potential therapies have focused on anti-inflammatories or disrupting TNFα signaling but have had either little success or adverse effects on other gastrointestinal organs (Gao 2012). IL-22, which has a cytoprotective role in hepatocytes, may be effective in preventing some the adverse effects of anti-inflammatory or anti-TNFα therapies (Park et al. 2011). Other potential therapeutics include inhibitors of MCP-1 and MIF to decrease infiltrating macrophages (Barnes et al. 2013; Mandrekar et al. 2011) and IL-1 receptor antagonists to inhibit inflammasomes (Petrasek et al. 2012). Some groups have taken strategies to enhance anti-inflammatory pathways rather than directly inhibiting inflammatory cytokines or chemokines. For example, PDE4 inhibitors increase the production of the cAMP, an important anti-inflammatory signal (McClain et al. 1998), while low molecular weight hyaluronic acid of a molecular weight of 35 kDa (HA35) normalized TLR4 signaling in Kupffer cells isolated from ethanol-fed rats and prevented ethanol-induced liver injury in mice (Saikia et al. 2017). HA35, via activation of CD44, decreased expression of importin α5 and reduced the translocation of p65 to the nucleus (Saikia et al. 2017).

Chemokines have also been considered as another potential target for therapeutic interventions in ALD. However, given the highly complex interplay between cells, chemokines, chemokine receptors, and disease stage in ALD, it is unlikely that directly targeting a specific chemokine or receptor would be effective. Moreover, chemokine gene families cluster at loci on chromosomes in both humans and mice, indicating stimuli that increase chemokine production could similarly affect many chemokines in the same family (Zlotnik et al. 2006). Therefore, potential therapeutic avenues for ALD focused on chemokines would ideally target upstream factors that lead to increased chemokine expression. Further, identification of circulating chemokines using systems approaches, for instance, could be developed into a new diagnostic tool for ALD.

A new avenue of therapeutics may target the regulation of miRNAs; miRNA expression is strongly dysregulated during the progression of ALD. For example, miR223, which inhibits pro-inflammatory IL-6 in neutrophils, is highly expressed in mouse

models of chronic ethanol feeding but interestingly is downregulated in alcoholic patients (Li et al. 2017). Loss miR223 increases liver injury in response to alcohol, so increasing miR223 expression may protect the liver from damage (He et al. 2017). miR-155, which is regulated by TLR4, is highly upregulated in Kupffer cells from chronic ethanol-fed mice and increases pro-inflammatory signals, making it a potential therapeutic target (Bala et al. 2016).

EVs are also prospective therapeutic tools, as the vesicles circumvent immune-mediated rejection, drug resistance antiporters, freely cross the blood-brain barrier, and can be used experimentally in vitro and in vivo (Hirsova et al. 2016). Innovative work in the liver fibrosis field highlights both the pathogenic and therapeutic potential of exosomes in ALD. Upregulation of connective tissue growth factor (CCN2) induces fibrogenesis through hepatic stellate cell activation. Two negative regulators of CCN2, miR-214 (Chen et al. 2014, 2015) and miR-199a-5p (Chen et al. 2016b), were shown to be decreased n exosomes following models of fibrosis and ethanol exposure. These exosomal miRs were found to be critical regulators of fibrogenesis, and furthermore, the transfer of exosomes derived from either quiescent cells or loaded with miR mimics prevented stellate cell activation and fibrogenesis.

5 Comparison Between Effects of Alcohol on Hepatic and Neural Immune Systems

Here we have reviewed the interactions between ethanol and immune functions in the liver. What is clear from studies done over the last decades is that both innate and adaptive immune function is critical for the maintenance of homeostasis in the healthy liver; immune activity serves to maintain the tolerogenic capacity of the liver and contributes to maintain normal responses to various stresses, as well as appropriate cellular turnover and repair. In response to heavy, chronic alcohol exposure, the finely tuned balance of pro- and anti-inflammatory functions is disrupted, leading to a chronic state of inflammation in the liver. Over time, this inability to resolve the inflammation contributes to the progression of liver disease (Wang et al. 2012).

Similar to the role of the innate immune response in the healthy liver, it is becoming evident that there is an active and complex neuroimmune system that serves to maintain a healthy central nervous system. Both cells of innate immune system (microglia, astrocytes), receptors (e.g., TLRs), and soluble mediators (e.g., cAMP, complement, TNF, IL1β) contribute to a wide range of physiological functions. For example, microglia play a major role in the development of the nervous system, as well as maintaining neuronal plasticity and synaptic pruning (Salter and Stevens 2017). Paralleling the impact of ethanol on the hepatic immune system, chronic, heavy alcohol exposure dysregulates neuroimmune activity, leading to a condition of neural inflammation (reviewed in other chapters of this volume by Crews et al. and Roberto et al.). Many of the same mediators, including HMGB1, IL1β, TNF, and multiple TLRs, contribute to both ethanol-induced inflammation in the liver and brain. However, it is not clear whether the mechanisms by which ethanol dysregulates neural vs hepatic immune responses are similar (Montesinos et al. 2016).

There is also a growing appreciation that neuroimmunity can also influence behaviors, including alcohol consumption (McCarthy et al. 2017) and depression (Wetsman 2017); however, it is not clear whether this is a physiological role for the neuroimmune system or a consequence of neuroinflammation. Future studies aimed at understanding the mechanistic parallels between ethanol-induced inflammation in the central nervous system and the liver may lead to therapeutic interventions that may target both the behavioral impact of neuroinflammation and the progression of alcohol-induced liver injury.

Grant Support This work was supported in part by NIH grants P50 AA024333, RO1 AA 023722 and U01 AA021890 to LEN, F32 AA024955 to KLP, and K99 AA025386 to RLM.

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Innate Immune Signaling and Alcohol Use Disorders

Leon G. Coleman, Jr. and Fulton T. Crews

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Abstract

Innate immune signaling is an important feature in the pathology of alcohol use disorders. Alcohol abuse causes persistent innate immune activation in the brain. This is seen in postmortem human alcoholic brain specimens, as well as in

The original version of this chapter was revised. A correction to this chapter is available at https://doi.org/10.1007/164_2018_193.

L. G. Coleman, Jr. (✉) · F. T. Crews
Bowles Center for Alcohol Studies, Department of Pharmacology, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC, USA

Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
e-mail: leon_coleman@med.unc.edu; fulton_crews@med.unc.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_92

primate and rodent models of alcohol consumption. Further, in vitro models of alcohol exposure in neurons and glia also demonstrate innate immune activation. The activation of the innate immune system seems to be important in the development of alcohol use pathology, as anti-immune therapies reduce pathology and ethanol self-administration in rodent models. Further, innate immune activation has been identified in each of the stages of addiction: binge/intoxication, withdrawal/negative affect, and preoccupation/craving. This suggests that innate immune activation may play a role both in the development and maintenance of alcoholic pathology. In this chapter, we discuss the known contributions of innate immune signaling in the pathology of alcohol use disorders, and present potential therapeutic interventions that may be beneficial for alcohol use disorders.

Keywords
Addiction · Alcohol · Neuroimmune · Treatment

1 Immune Signaling in the Brain

1.1 Immune Cells in the Brain

The brain has effective mechanisms for protection against infectious agents. Primarily, this is thought to occur via the maintenance of its "immune privileged" status by the physical protection of the blood–brain barrier. However, the brain also has resident immune defenses, which are primarily innate immune cells. In the brain the resident immune cells are primarily microglia and astrocytes. These cells are capable of recognizing and responding to viral, bacterial, and fungal pathogens. Microglia, astrocytes, and neurons contain innate immune signaling receptors and capabilities.

Microglia are often considered the resident macrophages of the brain. However, microglia are unique from peripheral macrophages. Microglia are the only myeloid cells that originate from yolk-sac progenitor cells (Sheng et al. 2015; Hoeffel et al. 2015). Microglia are formed early in embryonic brain development (E8 in mice) and maintain stable levels in adulthood through neuro-proliferation throughout the lifespan (Ginhoux et al. 2010). There remains debate about whether peripheral monocytes migrate into brain in normal physiological circumstances (Ginhoux et al. 2013). Both microglia and macrophages share multiple markers such as Cluster of Differentiation (CD)-45, CD11b, and ionized calcium-binding adapter molecule 1 (Iba-1). However, microglia-retain unique functions from peripheral monocytes, such as an involvement in synaptic pruning, debris clearance, and the regulation of adult neurogenesis (Salter and Stevens 2017; Kettenmann et al. 2011, 2013). Microglia transition from physiological "resting" state to activated states in response to infections, stressors, and drugs of abuse such as alcohol and cocaine (Beynon and Walker 2012; Streit 2002; Guo et al. 2015; Coleman et al. 2017; Qin et al. 2008; He and Crews 2008; Periyasamy et al. 2016). Microglial activation state is traditionally classified as M1 (pro-inflammatory) or M2 (anti-inflammatory), though their function is likely more complicated. The M1 activation state is defined by the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα),

interleukin (IL)-1β, and IL-6 with accompanying reactive oxygen species generation by inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate-(NADPH) oxidase expression. M2 state is defined by the release of "anti-inflammatory" cytokines such as IL-10 and IL-4. These activation states are a simplified framework to understand microglial activation. Alcohol and other drugs of abuse modulate microglial activation, contributing to disease pathology.

Astrocytes are also involved in neuroimmune responses in the brain (Farina et al. 2007). Astrocytes express immune receptors and release cytokines when activated (Jensen et al. 2013). This activation, known as reactive gliosis, can limit tissue damage in several contexts (Pekny and Pekna 2014). Astrocytes might also have pro- and anti-inflammatory states, similar to microglia (Jang et al. 2013), but this has yet to be fully elucidated. Astrocytes can be activated by microglia to release neurotoxic factors that damage neurons (Liddelow et al. 2017; Hashioka et al. 2015; Lee et al. 2013). We found that the expression of many immune genes and receptors are unchanged in brain after pharmacological depletion of microglia with the compound Plexicon (Walter and Crews 2017). Thus, astrocytes and neurons seem to express higher levels of immune mediators than previously believed. There continues to be debate regarding the exact immune function of astrocytes. In Sects. 1.3 and 2.3 specific immune receptors on astrocytes are discussed. This is currently being investigated with in vitro culture models as well as chemogenetic in vivo models. In addition to immune function, astrocytes are also involved in fluid homeostasis, metabolic support of neurons, and modulation of glutamate concentrations at the synapse (Khakh and Sofroniew 2015). Drugs of abuse such as alcohol and cocaine cause astrocyte activation (Periyasamy et al. 2016; Valles et al. 2004; Alfonso-Loeches et al. 2010). It is important to note that since microglia and astrocytes regulate synaptic plasticity, activation of immune signaling in these cells might alter synaptic firing and neuroplasticity.

Though glia (i.e., microglia and astrocytes) are considered the primary neuro-immune cells, neurons also seem to play a role in innate immune responses (Lawrimore and Crews 2017). Neurons can regulate glial responses through factors such as fractalkine, and also express many cytokine receptors, such as those for TNFα, IL-1β, IL-6, and the interferons (IFNs) (Khairova et al. 2009). Immune molecules have normal physiological roles in neurons that regulate synaptic firing and plasticity. For instance IL-1β modulates γ-aminobutyric acid (GABA) transmission in the central nucleus of the amygdala (Bajo et al. 2015a, b) and monocyte chemoattractant protein (MCP-1) increases dopamine release in the rat substantia nigra (Rostene et al. 2007). The effects of cytokines and chemokines on ethanol responses are discussed in Sect. 2. Thus, neurons contain and respond to immune signaling molecules. These cytokines and other immune signaling molecules not only regulate immune responses, but they also modulate synapses and neurocircuits.

1.2 Innate Immune Signaling Molecules as Modulators of Neurocircuitry

Increasing evidence from brain studies indicate that the neuroimmune system is involved in the regulation of brain function, apart from its role in response to

pathogens. Several immune signaling molecules have been found to regulate synaptic activity, learning, and memory (see Table 1). TNFα is considered a classic pro-inflammatory cytokine. However, in the brain, TNFα also regulates long-term potentiation (LTP). LTP is a form of plasticity that involves increased synaptic excitability following a burst of firing that is thought to reflect components of memory formation. TNFα is required for proper LTP in visual cortical slices from rats and mice (Sugimura et al. 2015), but disrupts LTP at higher concentrations (Tancredi et al. 1992). This results in behavioral dysfunction, with TNFα overexpressing mice having decreased performance on spatial learning and memory tasks (Aloe et al. 1999). TNFα also regulates synaptic strength in hippocampal neurons by increasing AMPA receptor surface expression (Beattie et al. 2002). The pro-inflammatory cytokine IL-1β also modulates LTP, promoting it at lower levels, and disrupting LTP at higher concentrations, similar to TNFα (Prieto and Cotman 2017; Prieto et al. 2015; Goshen et al. 2007). Pro-inflammatory chemokines macrophage inflammatory protein alpha (MIP-1α) and fractalkine/chemokine (C-X-C motif) ligand 1 (CX3CL1) also regulate synaptic plasticity and memory function (Marciniak et al. 2015; Bian et al. 2015). CX3CL1 is expressed in neurons and is an anti-inflammatory signal to microglia. CX3CL1 KO mice show impaired LTP, with exogenously added MIP-1α impairing LTP. These changes might be similar to those seen with IL-1β and TNFα where the dose response is critical for functions in LTP.

Traditional anti-inflammatory molecules also regulate synaptic plasticity. The anti-inflammatory protein transforming growth factor-β1 (TGF-β1) promotes LTP and object recognition memory (Caraci et al. 2015). Further, anti-inflammatory cytokines IL-4 and IL-13 regulate learning and memory. IL-4 and IL-13 knockout (KO) mice show learning and memory impairments (Brombacher et al. 2017; Derecki et al. 2010). Thus, since both cytokines and chemokines can regulate LTP and synaptic strength, neuroimmune signaling may actually be a form of neuroplasticity. In addition to LTP, cytokines can modulate inhibitory GABA and excitatory glutamatergic signaling. For instance, IL-1β modulates neuronal GABA

Table 1 Innate immune molecules involved in neuroplasticity

Neuroimmune molecule	Synaptic function
TNFα	LTP (Sugimura et al. 2015) Synaptic strength (Beattie et al. 2002)
Il-1β	LTP (Prieto and Cotman 2017; Prieto et al. 2015; Goshen et al. 2007) GABA transmission in CeA (Bajo et al. 2015a, b)
Il-4	Learning and memory (Brombacher et al. 2017; Derecki et al. 2010)
Il-13	Learning and memory (Brombacher et al. 2017; Derecki et al. 2010)
CX3CL1	LTP (Bian et al. 2015)
MIP-1	LTP (Marciniak et al. 2015)
TGFβ	Promotes LTP (Caraci et al. 2015)
CXCL16	GABA transmission in hippocampus (Di Castro et al. 2016)
HMGB1	Excitatory signaling (Liang et al. 2014; Maroso et al. 2011)

transmission in the central amygdala (CeA), decreasing the amplitude of evoked inhibitory postsynaptic potentials (eIPSPs) and differentially modulating inhibitory postsynaptic currents (mIPSCs) (Bajo et al. 2015a, b). The chemokine CXCL16 also modulates GABA transmission in the hippocampal CA1 region by increasing the frequency of mIPSCs via increased presynaptic GABA release (Di Castro et al. 2016). Thus, in the brain, immune signaling molecules act as neuromodulators. Many of these molecules are altered by drugs of abuse which could lead to altered synaptic activity and behavior.

1.3 Endogenous Toll-Like Receptor (TLR) Agonists and Pattern Recognition Receptors

A key feature of the innate immune system is the recognition of foreign pathogens and endogenous damage-associated molecules. Specialized groups of receptors have been identified that recognize specific molecular signatures of pathogen-associated molecular patterns (PAMPs) from bacteria, fungi, viruses, or endogenous damage-associated molecular patterns (DAMPs). These pattern recognition receptors (PRRs) are grouped into five different classes: toll-like receptors (TLRs), C-type lectin receptors, nucleotide binding domain receptors (leucine-rich repeat containing or NOD-like receptors), RIG-I-like receptors (RLRs), and AIM 2-like receptors (Brubaker et al. 2015). TLRs are the best characterized PRR and are involved in numerous disease states including alcohol and drug addiction (Periyasamy et al. 2017; Bachtell et al. 2015; Northcutt et al. 2015; Crews et al. 2017). Ten TLRs are known in humans and 12 in mice (Brubaker et al. 2015). Ligands for TLRs include a variety of molecules from bacterial endotoxin to mammalian high-mobility group box protein 1 (HMGB1) and heat shock proteins (Vabulas et al. 2002). The ability of TLRs to recognize DAMPs creates the situation known as "sterile inflammation." Sterile inflammation occurs when innate immune signaling is activated in the absence of an invading organism. Since immune signaling can modulate neurocircuitry, functional consequences of sterile inflammation are likely critical. Indeed, TLR signaling has been found to play a role in alcoholism and other neurological conditions. Table 2 illustrates some key TLRs, their foreign PAMP, endogenous DAMP, and associated neurological diseases.

TLRs contain an N-terminal extracellular leucine-rich repeat sequence and an intracellular toll/interleukin-1 receptor/resistance motif (TIR) (Takeuchi and Akira 2010). TLR signaling utilizes key adapter proteins to initiate a signaling cascade upon ligand recognition. Each of the TLRs, minus TLR3, utilizes the MyD88 adapter protein complex. TIRAP/MyD88 complex formation leads to activation of the IL-1 receptor-associated kinases (IRAKs) and the TNF receptor-associated factor 6 (TRAF6) leading to IκB kinase (IKK) and mitogen-activated protein kinase (MAPK) activation, followed by activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activated protein-1 (AP-1) transcription factors respectively. Endosomal TLRs (i.e., TLR3, TLR7, and TLR9) cause activation of the interferon regulator factors (IRFs), transcription factors that lead to

Table 2 Toll-like receptors (TLRs) implicated in alcoholism and neurological diseases

TLR	Foreign immunogen	Endogenous TLR ligand	Neuropsychiatric disease
2	Bacterial di- and tri-aceylated polypeptides (Buwitt-Beckmann et al. 2006) Gram (+) lipoglycans (Blanc et al. 2013)	α-synuclein (Kim et al. 2013)	Alcoholism Parkinson's disease (Kim et al. 2013)
3	dsRNA	Stathmin (Bsibsi et al. 2010)	Alzheimer's disease (Jackson et al. 2006) Multiple sclerosis (Bsibsi et al. 2010)
4	Bacterial endotoxin Peptidoglycans	HMGB1 (Park et al. 2004) HSPs 60, 70/72 (Vabulas et al. 2002)	Alcoholism (Crews et al. 2013) Cocaine abuse Stroke, traumatic brain injury Chronic pain
7	ssRNA (Lehmann et al. 2012b)	miRNAs let-7 (Lehmann et al. 2012a) and miR-21 (Yelamanchili et al. 2015)	Alcoholism (Coleman et al. 2017) Alzheimer's disease (Lehmann et al. 2012a) Chronic pain (Park et al. 2014)

interferon induction (see Fig. 1). These key transcription factors subsequently induce the expression of several pro-inflammatory cytokines and mediators to perpetuate the immune response. The involvement of these transcription factors in addiction is detailed in subsequent sections. Selected key TLRs, cytokines, and chemokines are illustrated in Fig. 1, as well as their expression on different brain cell types. The exact TLR signaling pathways in brain cells type is an ongoing work, as there are some differences between brain and peripheral immune cells, in which TLR signaling was initially described. Both microglia and astrocytes express TLRs; however, the effects of activation of specific TLRs may vary between cell types. For instance, there is question over whether astrocytes have TLR4 responses, which may depend on culture conditions (Gorina et al. 2011; Barbierato et al. 2013). Astrocytes do, however, have strong TLR3 responses (Serramia et al. 2015) and can release an assortment of immune mediators. Further, responses in neurons are less well understood. Neurons appear to be capable of activating NF-κB in some settings but not others (Mao et al. 2009). Neuronal NF-κB might regulate plasticity, learning, and memory (Lawrimore and Crews 2017; Kaltschmidt and Kaltschmidt 2015) in glutamatergic and GABAergic neurons. Dorsal horn spinal neurons (Bai et al. 2014) have activated NF-κB and different neuronal cell lines exhibit NF-κB dependent regulation of μ-opioid receptors (Borner et al. 2012; Wagley et al. 2013). Though the exact downstream signaling pathways in neurons needs to be studied in each specific context, TLRs undoubtedly play important roles in normal neuronal

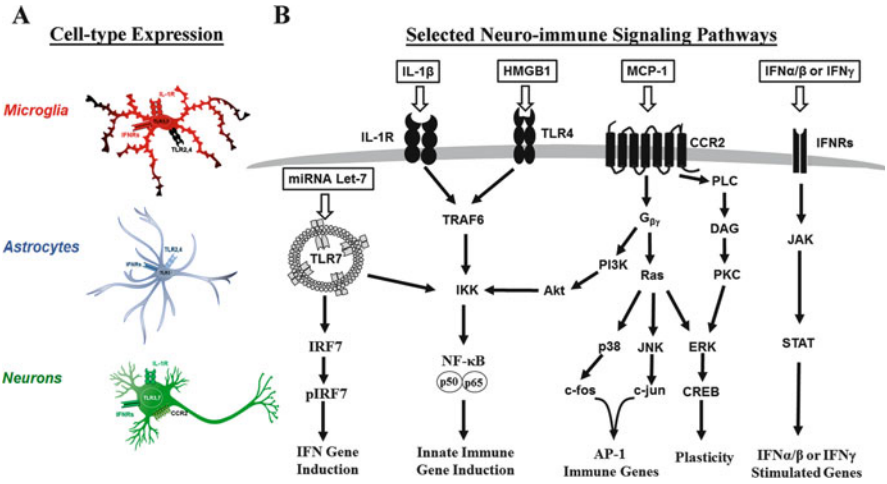

Fig. 1 Selected toll-like receptors (TLRs), cytokine (IL-1β), and chemokine (MCP-1) signaling pathways in brain cells relevant to alcoholism. Selected immune signaling pathways that are involved in alcohol use pathologies are illustrated. (**a**) The three primary neuroimmune cell types – microglia, astrocytes, and neurons – are illustrated with selected immune receptors that are relevant to alcohol use disorders. Several TLRs are implicated in microglial and astrocyte activation including TLRs2–4 and 7. Microglia also contain the IL-1β receptor (IL-1R) and interferon (IFN) receptors. Astrocytes contain TLRs2–4 as well as IFN receptors. Neurons have TLR3 and 7 responses in the context of alcohol use disorders, as well as the MCP-1 receptor (CCR2) and IFN receptors. (**b**) Simplified versions of the signaling cascades for immune receptors that are relevant to alcoholism are shown. The TLR7 pathway has been shown to be involved in alcoholic hippocampal neurodegeneration, and can lead to IFN gene induction via IRF7 as well as NFκB-mediated immune gene induction. Both the IL-1R and surface TLRs such as TLR4 share the same downstream signaling pathway leading to NFκB-mediated immune gene induction. The chemokine MCP-1 regulates ethanol self-administration and is a G-protein coupled receptor that can result in AP-1-mediated immune gene induction, NFκB activation, or neuronal plasticity via CREB signaling. The interferon receptors (IFN) are on all three cell types and are associated with depressive phenotypes. These activate the JAK/STAT signaling pathway to result in interferon response gene expression. See Lehmann et al. (2012a, b), Coleman et al. (2017), Fernandez-Lizarbe et al. (2009, 2013), Blanco et al. (2005), Montesinos et al. (2015), Narayanan and Park (2015), and Bose and Cho (2013)

function. For instance TLR3 and TLR8 regulate axonal or neurite outgrowth, respectively (Cameron et al. 2007; Ma et al. 2006). TLR7 activation can cause neurodegeneration (Coleman et al. 2017; Lehmann et al. 2012a). The presence of physiological, non-pathologic, and non-damage associated TLR signaling in brain argues that endogenous ligands for these receptors play key roles in normal physiology.

Since endogenous TLR agonists play key roles in normal brain physiology, a better understanding of these ligands is required. Traditionally called DAMPs (i.e. damage associated molecular patterns), in the brain these agonists may have normal biological functions. However, in the context of disease states, these DAMPs subsequently lead to further TLR activation by enhanced induction of their receptors. One DAMP that has been found to play a role in alcohol addiction in

particular is the protein high-mobility group box 1 (HMGB1). HMGB1 is a nuclear chromatin binding protein that is released during bacterial infection, cellular stress, or damage. After its release HMGB1 can bind directly to TLR4 or receptor for advanced glycation endproducts (RAGE) receptors (Muller et al. 2004; Janko et al. 2014). However, HMGB1 also can modulate activity of several other TLRs such as TLR3, 7, and 9 (Yanai et al. 2009). HMGB1 might also regulate synaptic firing, as it is released just prior to hyperexicitable states, such as seizures, to modulate glutamatergic signaling (Maroso et al. 2011). The role of HMGB1 in the pathology of alcoholism is further described in Sect. 2.3. Neuroimmune activation and neuronal signaling might be interconnected by the release of DAMP such as HMGB1 in addition to cytokines. Thus, understanding DAMPs and cytokines play key roles in the neuroimmune system that may regulate neuron signaling and brain function.

2 Neuroimmune Activation in the Pathology of Alcoholism

2.1 The Natural History of Alcohol Use Disorders

Neuroimmune activation in addiction has been elucidated by several laboratories, with several reviews on the topic (Crews et al. 2011, 2015, 2017; Neupane 2016; Ballester et al. 2017; Montesinos et al. 2016; Jacobsen et al. 2016; Crews and Vetreno 2014, 2016; Vetreno and Crews 2014; Most et al. 2014; Ray et al. 2014; Loftis and Janowsky 2014; Cui et al. 2014; Mayfield et al. 2013). Alcoholism develops progressively over the course of an individual's lifespan. This often begins during adolescence as drug exploration (Fig. 2). The age of drinking onset is strongly associated with the risk of developing an alcohol use disorder in adulthood (Dawson et al. 2008; Grant and Dawson 1998). Adolescence is a key developmental period during when maturation of frontal cortical structures regulating cognitive function and decision-making occurs (for review see Crews et al. 2016). A key cognitive feature in the progression to addiction is a loss in frontal-cortical-mediated executive functions. This includes motivation, planning, goal setting, and behavioral flexibility. Binge drinkers show impairments in executive functioning tasks (Townshend and Duka 2003; Weissenborn and Duka 2003; Crews and Boettiger 2009). Reversal learning (the ability to change previously learned behaviors) is impaired in both human alcoholics (Fortier et al. 2008; Jokisch et al. 2014) and cocaine addicts (Stalnaker et al. 2009). Mice and rats also have long-lasting impairment of reversal learning following binge ethanol (Obernier et al. 2002; Coleman et al. 2011; Badanich et al. 2011) or cocaine (Calu et al. 2007; Schoenbaum et al. 2004). Prefrontal cortical regions regulate these behaviors in concert with striatum and amygdala (Izquierdo et al. 2016) through reciprocal glutamatergic connections. Glutamatergic dysfunction is a well-known finding in alcoholism and drug abuse. Neuroimmune gene induction has been found to contribute to glutamatergic hyperexcitability in the frontal cortex and to impair executive function (Crews et al. 2006, 2011). Thus, neuroimmune activation might contribute to the development of cognitive dysfunction due to altering prefrontal cortical to limbic circuitry, though

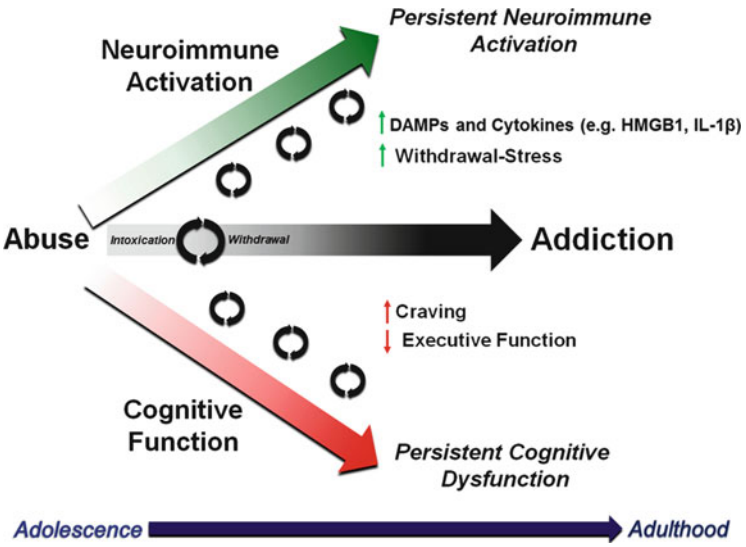

Fig. 2 Multiple drug exposures amplify neuroimmune signals and cognitive decline. The natural history of alcohol use disorders involves a progression from adolescence into adulthood of recurrent cycles of binge intoxication and withdrawal. Neuroimmune signaling is amplified with each cycle, as cognitive function progressively worsens

this needs to be elucidated. Recurrent immune activation across the lifespan, from adolescence into adulthood, may contribute to lasting cognitive dysfunction in alcoholism through these and other mechanisms.

The pathology of addiction has been described as a cycle consisting of three recurring stages (Cui et al. 2015; Volkow et al. 2016). This includes cycling through binge/intoxication, withdrawal and negative affect, craving and preoccupation, which leads to recurrent binge intoxication (Koob and Volkow 2010) (Fig. 3). Specific neurocircuits are involved in the regulation of behavioral phenotypes associated with each stage in the cycle (Cui et al. 2015; Koob and Volkow 2016). For example, the binge/intoxication stage involves reward pathways that involve dopaminergic and opiod signaling originating in the basal ganglia. This includes the dorsal striatum (caudate nucleus and putamen), the ventral striatum (nucleus accumbens), globus pallidus, ventral pallidum, and substantia nigra. The withdrawal/negative affect stage involves circuits that regulate stress, fear, and anxiety. This includes pathways such as the amydala-nucleus accumbens and ventral tegmental area (VTA). The craving/preoccupation stage is likely associated with prefrontal cortical dysfunction mentioned above. The complexity of the dysfunction in these circuits is significant, as reciprocal and overlapping connections cause interplay between the different circuits typically associated with each individual stage. Thus, each stage is connected, and the dysfunction of several circuits is likely linked. Interestingly, neuroimmune signaling has been implicated in each of the three stages of addiction, though there remains much to be further understood about

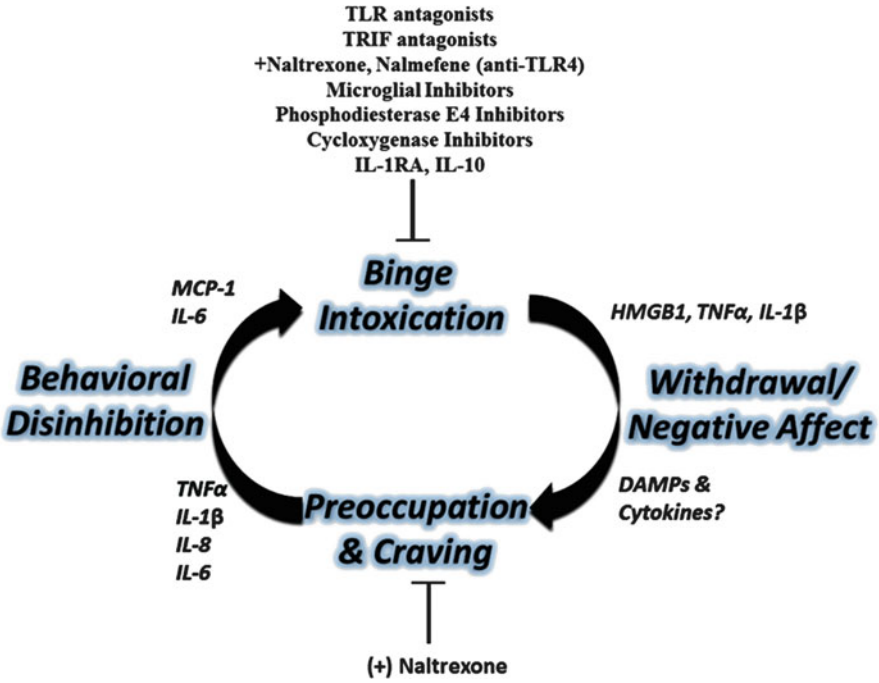

Fig. 3 Neuroimmune contributions to the cycle of addiction. The three main stages of the cycle of addiction-binge/intoxication, withdrawal/negative affect, and preoccupation/craving-each have neuroimmune contributions. Multiple neuroimmune interventions reduce alcohol self-administration in rodent models. Binge intoxication causes the induction of several immune signaling molecules such as HMGB1, TNFα, and IL-1β. Neuroimmune molecules might also mediate some of the negative affect seen during withdrawal. The TLR4 antagonist (+)-Naltrexone reduces alcohol-induced conditioned-place preference (a feature of craving), and several immune molecules in plasma have been associated with craving in human alcoholics. See Marshall et al. (2016a), Agrawal et al. (2011), Jacobsen et al. (2018), McCarthy et al. (2017), Blednov et al. (2014), Wang et al. (2016), and Montesinos et al. (2017)

the precise effects of neuroimmune activation on specific circuitry. The majority of the studies investigating neuroimmune contributions in vivo have been done on the binge/intoxication stage, leaving much to be examined in the other stages, especially the withdrawal/negative affect stage. However, several inflammatory mediators have been found to play important roles at different stages, and certain neuroimmune therapies are effective in reducing ethanol consumption in rodent models.

2.2 Neuroimmune Activation in the Stages of Addiction

2.2.1 Binge/Intoxication Stage

The binge/intoxication stage is perhaps the most studied stage involving neuroimmune activation in alcoholism. Multiple immune regulating interventions

have been found to alter ethanol consumption in rodents, suggesting that neuroimmune activation can drive ethanol consumption. A genetic analysis found that high ethanol drinking rodents had increased expression of NF-κB and other pro-inflammatory genes (Mulligan et al. 2006). A significant amount of work has been done surrounding TLR4. Sensitization of TLR4 responses by injection of the TLR4 agonist LPS increases ethanol self-administration in a two-bottle choice paradigm in mice (Blednov et al. 2011). Selective knockdown of TLR4 in the central amygdala decreases ethanol self-administration in alcohol-preferring strain of rats known as p-rats (Liu et al. 2011). Knockdown of TLR4 in the ventral palladum, however, had no effect, suggesting brain regional specificity of TLR4 involvement in self-administration. Intracerebroventricular injection of the chemokine MCP-1 also increases alcohol self-administration in rats (Valenta and Gonzales 2016). Inhibition of several traditional pro-inflammatory signaling has also been shown to reduce ethanol self-administration. Ablation of many key neuroimmune genes including IL-6, Ccr2, MCP-1, and Ccl3 decreases ethanol consumption (Blednov et al. 2005, 2012). The IL-1 receptor antagonist and the anti-inflammatory IL-10 both reduce alcohol self-administration when injected into the basolateral amygdala (Marshall et al. 2016a, 2017). Furthermore, IL-1β inhibition in the VTA also prevents cocaine-induced dopamine release in the nucleus accumbens (Northcutt et al. 2015), suggesting these signals are involved in other drugs of abuse. Broad acting inhibitors of microglial activation also reduce ethanol consumption in rodents. Minocycline, a tetracycline antibiotic and microglial inhibitor, reduces ethanol self-administration (Agrawal et al. 2011) as well as conditioned place preference after cocaine exposure (Northcutt et al. 2015). These studies suggest that neuroimmune signaling plays a role in the rewarding properties of alcohol, or in the early stages of dependence. However, translating rodent drinking studies to humans can be challenging, as most rodent studies are not in dependent models. Further, in addicted humans, the rewarding properties of the drug seem to shift from the drug itself to cues associated with the drug (Koob and Volkow 2010). Therefore, future work is needed in models that have more features of ethanol dependence. Nonetheless, neuroimmune signaling is clearly involved in ethanol consumption and warrants further investigation.

2.2.2 Craving/Preoccupation Stage

Regarding the craving/preoccupation stage, innate immune activation both in the brain and periphery seems to be important. In human alcoholics, certain key inflammatory mediators are elevated in plasma that correlate with alcohol craving. This includes TNFα (Heberlein et al. 2014) (which also correlated with the severity of alcoholism), IL-1β, IL-6, and IL-8 (Heberlein et al. 2014; Leclercq et al. 2014). Several of these cytokines such as TNFα, IL-6, and IL-1β can cross the blood–brain barrier to exert effects on the brain (Banks et al. 1994, 1995), and could drive craving in humans. However, they might also regulate craving via modulation of systemic stress responses. Recently, the systemic administration of the TLR4 antagonist (+)-naltrexone was found to reduce alcohol-induced conditioned place preference, which is likely a feature of craving (Jacobsen et al. 2018). Further, (+)-naltrexone reduces cue-induced heroine seeking (Theberge et al. 2013). These findings suggest

that immune interventions may be effective at reducing alcohol and drug-associated craving or preoccupation. More work needs to be done to differentiate central from peripheral immune involvement in the craving/preoccupation stage. This can be challenging in animal models, as craving is a subjective measure in humans. However, assays such as conditioned place preference can identify features of craving and should be utilized to further dissect the neuroimmune contribution.

2.2.3 Withdrawal/Negative Affect and Stress

The withdrawal/negative affect stage of addiction is a key feature in the maintenance of addiction (Koob and Le Moal 2005). Negative affective states include stress, anxiety, and dysphoria. Neuroimmune signaling seems to be involved in this stage as well. Several inflammatory cytokines including TNFα, MCP-1, as well as inflammatory transcription factors are increased in whole brain during withdrawal 24 h after a 10-day chronic binge ethanol exposure in mice (Qin et al. 2008). Transcription factors such as NFκB and CEBP are increased in the rodent hippocampus and amygdala respectively during withdrawal from ethanol (Freeman et al. 2012; Qin and Crews 2012a). Further, intracerebroventricular (ICV) injection of TNFα, IL-1β, and MCP-1 sensitize to anxiety-like behavior during alcohol withdrawal (Breese et al. 2008), suggesting that these cytokines contribute to withdrawal-associated negative affect. Interestingly, rhesus macaques that show heavy or binge drinking patterns during adolescence have elevated levels of MCP-1 in the plasma across the time of alcohol use (Fig. 4). MCP-1 might cross the blood–brain barrier and exert central effects, or it could alter stress responses. Stress pathways are critical in the negative affect stage. Neuroimmune activation is strongly associated with activation of stress pathways and may be a critical feature of modulating negative affect. Prior stress sensitizes microglia to inflammation in an HMGB1-dependent manner (Weber et al. 2015). Ethanol sensitizes microglia, increasing microglial markers such as CD11b and Iba1 (Fernandez-Lizarbe et al. 2009; Qin and Crews 2012b), priming the microglial response to subsequent systemic inflammatory responses (Qin and Crews 2012b). Further, TLR4 activation alters serotonin transporter (SERT) function to increase depressive behavior (Zhu et al. 2010). Chronic restraint stress causes microglia activation throughout the brain (Tynan et al. 2010) and leads to depression-like behavior. The pro-inflammatory cytokines TNFα, IL-6, and IL-1β contribute to the pathologies of mood disorders (Bhattacharya and Drevets 2017; Bhattacharya et al. 2016). Stress can also result in NF-κB activation. Psychosocial stress in humans drives NF-κB activation in blood monocytes (Bierhaus et al. 2003). Restraint stress in rodents causes NF-κB activation in rodents with production of TNFα and other pro-inflammatory prostaglandins (Bierhaus et al. 2003; Madrigal et al. 2002). Ethanol itself activates NF-κB in rat and mouse brain (Qin and Crews 2012a; Ward et al. 1996), and human astrocytes (Davis and Syapin 2004), and can interact with stress to enhance immune responses. On the other hand, agents that inhibit microglial activation can prevent the onset of depression-like behavior (Tynan et al. 2010; Frank et al. 2007; Wohleb et al. 2011; Kreisel et al. 2014). Thus, it is clear that there is overlay between stress and alcohol effects on glia, via immune mechanisms. However, the exact role of neuroimmune activation during

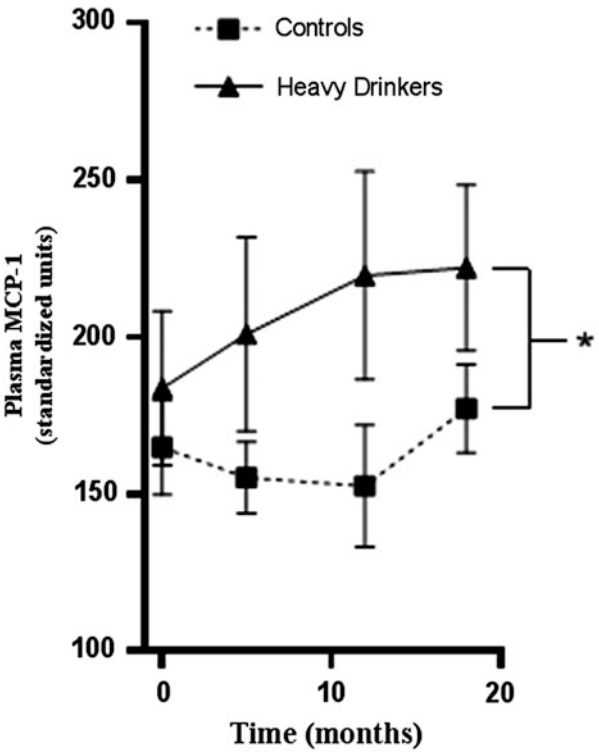

Fig. 4 Elevated plasma MCP-1 in heavy-drinking adolescent rhesus macaques. Adolescent rhesus macaques had free access to either water or ethanol for 18 months. MCP-1 was measured by cytokine multiplex. Ethanol drinkers showed increased plasma MCP-1 across the exposure period: 2-way ANOVA $F(1,38) = 4.250$, $^*p < 0.05$. Courtesy K. Grant et al., The Monkey Alcohol and Tissue Research Resource (MATRR)

ethanol withdrawal/negative affect needs to be clearly delineated. Further, it remains unclear exactly which immune mediators are involved in the negative affect associated with withdrawal. Identification of these mediators could offer important therapeutic options to help individuals suffering from negative affect during withdrawal and abstinence, and hopefully improve remission rates.

2.3 Neuroimmune Signaling in Alcoholism: TLRs and Endogenous TLR Agonists

The identification of neuroimmune activation in alcoholism has been supported by findings in postmortem human alcoholic tissue, rodent and cell culture experiments. Microglial and astrocytic markers are upregulated in postmortem human alcoholic brain (He and Crews 2008; Rubio-Araiz et al. 2017). Also, other immune markers are increased such as TLR2, TLR3, TLR4, TLR7, MCP-1, and HMGB1 (He and Crews 2008; Crews et al. 2013). Studies in vivo also find increased expression of TLRs 2–4, 7 and HMGB1 in different brain regions with associated NF-κB activation and cytokine induction (Crews et al. 2013; Coleman et al. 2017; Lippai et al. 2013). In vitro studies also find that ethanol activates brain cells. Microglial cell cultures find that ethanol causes activation, increasing expression of TNFα, IL-1β,

iNOS, and NADPH oxidase (Qin et al. 2008; Fernandez-Lizarbe et al. 2009; Qin and Crews 2012b). Ethanol causes NF-κB activation in neurons in brain slice culture (Coleman et al. 2017; Zou and Crews 2006) and in vivo (Ward et al. 1996) as well as increased NF-κB–DNA binding (Crews et al. 2006; Zou and Crews 2006). This leads to induction of proinflammatory cytokines (TNFα, IL-1β, and IL-6) (Qin et al. 2007), proinflammatory oxidases (inducible nitric oxide synthase (Alfonso-Loeches et al. 2010; Zou and Crews 2010), COX (Alfonso-Loeches et al. 2010; Knapp and Crews 1999), and NOX (Qin et al. 2008)), and proteases (TNFα-converting enzyme [TACE] and tissue plasminogen activator [tPA]) (Zou and Crews 2010). In addition to NF-κB activation, ethanol also causes IRF3 activation in both neuronal and microglial cell cultures (Lawrimore and Crews 2017). Astrocyte cell cultures similarly show activation with ethanol (Alfonso-Loeches et al. 2010; Franke 1995). Ex vivo brain slice cultures agree, showing immune activation with ethanol exposure (Zou and Crews 2010, 2012, 2014). Thus, ethanol can directly cause neuroimmune activation, in the absence of peripheral immune involvement. Further, each of these studies shows that ethanol neuroimmune induction involves TLR activation.

Activation of TLRs is a central feature in the neuroimmune responses to ethanol. This leads to pro-inflammatory transcription factor activation (e.g., NF-κB, AP-1, IRFs) and further amplification of immune responses. TLR4 has been the best studied TLR in alcohol-induced neuroimmune signaling (Alfonso-Loeches et al. 2010). TLR4 KO mice and TLR4 KO glia are protected from many features of the neuroimmune activation by ethanol. This includes protection from glial cell activation, NF-κB activation, caspase-3 cleavage, anxiety-like behavior, and memory impairment (Valles et al. 2004; Alfonso-Loeches et al. 2010; Fernandez-Lizarbe et al. 2009; Pascual et al. 2011; Blanco et al. 2005). TLR4 siRNA in cultured astrocytes (or siRNA to critical TLR adaptor proteins MD-2 and CD14) prevents ethanol induction of NF-κB (Blanco et al. 2005). Ethanol-preferring p-rats have increased expression of TLR4 in the VTA (June et al. 2015), with TLR4 expression being regulated by GABA(A)α2 receptor (Liu et al. 2011) and the stress regulating corticotropin-releasing factor (CRF) (June et al. 2015). TLR4 activation is also involved in cocaine and heroin abuse (Periyasamy et al. 2017; Northcutt et al. 2015; Theberge et al. 2013) suggesting a broader role in addictive pathologies. Though TLR4 is the best studied TLR in alcohol abuse, it is important to recognize that several TLRs are increased in the postmortem human alcoholic brain (TLRs 2, 3, 4, and 7) that show pathologic roles in vitro. Accordingly, TLR2 KO microglia are protected from ethanol-immune induction (iNOS and MAPK) (Fernandez-Lizarbe et al. 2013). Ethanol causes increased expression of TLR3 in neurons and TLR7, and TLR8 in both neuronal and microglial cell lines (Lawrimore and Crews 2017). Further, TLR7 has recently been identified as important in ethanol-induced hippocampal neurodegeneration (Coleman et al. 2017). Thus, several TLRs are important in ethanol-induced neuroimmune induction. The exact contribution of each TLR as well as the interplay between TLRs remains unclear. However, it does appear that the involvement of multiple TLRs and the induction of multiple immune transcription factors lead to amplified and integrated responses.

It is clear that TLR signaling is key in neuroimmune responses for ethanol. However, the brain is sterile under normal operating conditions. This suggests that endogenous TLR-agonists (also known as DAMPs) mediate TLR responses to ethanol. Indeed, this has been found to be true in the cases of TLR4 and TLR7. The endogenous TLR4 ligand, HMGB1, has been found to be a critical immune mediator in alcoholism. Postmortem human alcoholic brain shows HMGB1 is increased in several brain regions, which correlates with lifetime alcohol consumption (Coleman et al. 2017; Crews et al. 2013; Vetreno and Crews 2012). Rodent studies also find that ethanol administration increases HMGB1 in cortex and cerebellum (Crews et al. 2013; Lippai et al. 2013). In response to ethanol, microglia release HMGB1 (Coleman et al. 2017; Lawrimore and Crews 2017; Crews et al. 2013; Zou and Crews 2014). HMGB1 inhibition in vitro and in vivo protects against cytokine induction by ethanol (Zou and Crews 2014; Whitman et al. 2013). Methamphetamine also induces neuroimmune activation via HMGB1 induction both in vivo and in vitro (Frank et al. 2016), suggesting other drugs of abuse may also involve HMGB1 release. Ethanol also causes TLR7 activation through the release of its endogenous agonist. TLR7 is a single-stranded RNA virus sensing TLR that has also been found to bind the endogenous miRNA let-7b when it is present in microvesicles (Lehmann et al. 2012a). Ethanol causes the secretion of the TLR7 agonist miRNA let-7b in microvesicles leading to TLR7-mediated neurodegeneration (Coleman et al. 2017). Let-7 isoforms have been shown previously to be increased in postmortem human alcoholic brain as well as in chronic ethanol models in rodents (Lewohl et al. 2011; Nunez et al. 2013). Interestingly, HMGB1 served as a chaperone for let-7b, possibly mediating its vesicular secretion. Indeed, HMGB1 is critical for immune responses of each of the endosomal TLRs (i.e., TLRs 3, 7, and 9) (Yanai et al. 2009). HMGB1 might represent a critical mediator for the induction of multiple TLRs in the context of alcohol addiction. These endogenous agonists, and perhaps others that have yet to be identified, may serve as novel targets against the neuroimmune activities of ethanol. More work is necessary to identify the precise functions of these agonists within specific brain circuits and stages of addiction.

2.4 Neuroimmune Contribution to the Progression to Addiction

The persistent activation of the neuroimmune system by alcohol abuse seems to contribute the development of addiction (Crews et al. 2015, 2017; Vetreno and Crews 2014; Crews and Vetreno 2016). Binge drinking during the adolescent developmental period has long-lasting behavioral, functional, mood, and cognitive effects (Crews et al. 2016; Coleman et al. 2011; Vetreno and Crews 2012). A key feature of the neuroimmune activation in alcoholism that supports this hypothesis is the persistent upregulation of immune signals. The persistent upregulation of key immune molecules seen in postmortem human alcoholic brain tissue and rodents, and their correlation with years of drinking links the degree of immune activation with the progression of disease. Neuroimmune markers in alcoholics not only correlate with lifetime alcohol consumption and age of drinking onset (Crews

et al. 2013; Coleman et al. 2017; Crews and Vetreno 2016; Vetreno and Crews 2012; Vetreno et al. 2013), but some remain elevated during prolonged abstinent periods (Crews et al. 2013; Coleman et al. 2017; Vetreno and Crews 2012). Binge ethanol causes persistent upregulation of neuroimmune molecules including TLR3, TLR4, HMGB1, RAGE, etc. (Vetreno and Crews 2012; Vetreno et al. 2013). Further, ethanol sensitizes TLRs to future activation by their agonists (Qin et al. 2008; Qin and Crews 2012b). The recurrent and persistent induction of pro-inflammatory transcription factors by repeated alcohol might result in a chronic inflammatory state in the brain (see Fig. 5). This is supported by the increased innate immune markers in the postmortem brains of human alcoholics (Crews et al. 2013; Coleman et al. 2017), as well as the upregulation of NF-κB target genes in alcoholics (Okvist et al. 2007). Thus, chronic alcohol seems to shift the allosteric set point of immune activation in the brain. This is likely a result of multiple cycles of intoxication and withdrawal over time. It is well established that repeated cycles of binge and withdrawal amplify alcohol induced pathologies and behavioral dysfunction (Breese et al. 2005; Marshall et al. 2016b). Repeated neuroimmune induction may contribute

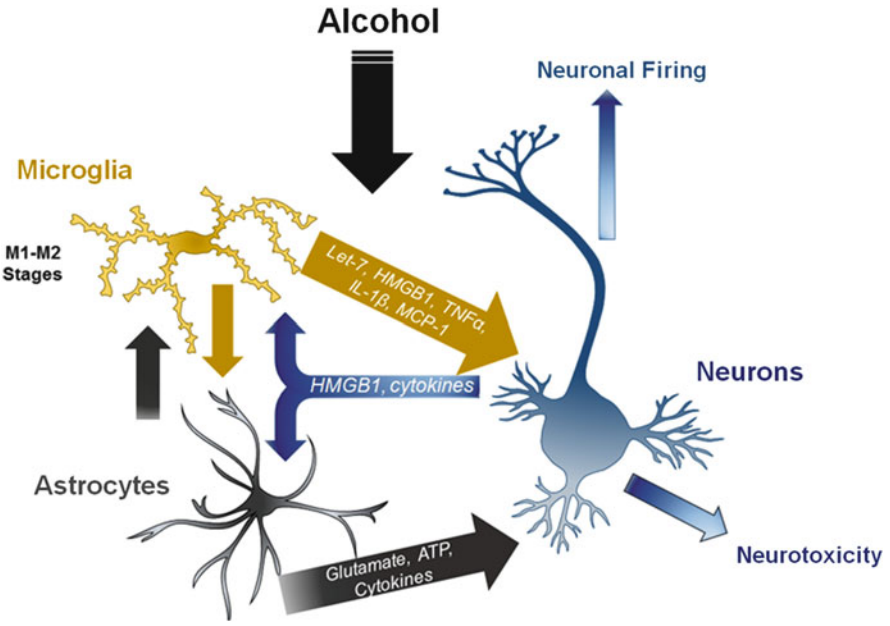

Fig. 5 Neuron-glia cell–cell interactions in neuroimmune responses to alcohol. Neuronal-glial interactions underlie neuroimmune signaling in alcohol use disorders. Microglia release factors such as microRNA let-7, HMGB1 and cytokines that can cause either neurotoxicity or altered neuronal activity. Microglia and astrocytes likely release factors that alter each other's activation status. Astrocytes modulate glutamate and ATP levels that affect neuronal signaling and vitality. Neurons release factors such as HMGB1, fractalkine, and cytokines that can modulate microglial and astrocyte activation. See Liddelow et al. (2017), Lee et al. (2013), Lawrimore and Crews (2017), Crews et al. (2013), Coleman et al. (2017), and Zou and Crews (2014)

to this amplification of pathology. The aforementioned effects of neuroimmune signals on plasticity suggest that changes in plasticity might be driven by repeated neuroimmune activation. Each acute inflammatory insult due to an alcohol binge may be additive, further increasing the "neuroimmune baseline." Future work should investigate how the recurrent amplification of neuroimmune responses corresponds to known changes associated with the transition from alcohol abuse to addiction.

2.5 Neuroimmune Basis of Addiction: An Ongoing Investigation

Much work has been presented in this chapter that supports a neuroimmune contribution to the development of alcoholism. However, much remains unknown. Particularly, the interactions between the immunocompetent brain cells – neurons, microglia, and astrocytes – need to be further investigated. These interactions could have very significant functional consequences. For example, a region-specific hyperglutamatergic state has been demonstrated in both alcohol and drug addiction (Reissner and Kalivas 2010), with astrocytes being key regulators of synaptic glutamate levels. Ethanol exposure induces NF-κB activation in astrocytes leading to increased expression of pro-inflammatory genes (Zou and Crews 2006, 2010; Pascual et al. 2007) and impaired astrocyte glutamate transport (Zou and Crews 2005). Increased extracellular glutamate levels causes enhanced neuronal excitation, microglial activation, and excitotoxicity (Zou and Crews 2006; Ward et al. 2009). TLR4 activation is involved in this interaction, as TLR4 KO mice are protected from the ethanol induced-hyperglutamatergic signaling and the associated neurotoxicity (Alfonso-Loeches et al. 2010; Knapp and Crews 1999). The tripartite synapse is composed of three immunocompetent cells, thus the interactions between these cells must be elucidated. In Fig. 5, we illustrate some of the known cell–cell interactions between neurons and glia regarding DAMPs (e.g., HMGB1 and miRNA let-7), cytokines (e.g., TNFα, IL-1β, and MCP-1), and glutamate. Several TLRs are involved in the alcohol-induced neuroimmune activation. There remains a paucity of understanding how these TLRs interact in the context of alcoholism, and at which stage of disease TLR antagonism might be effective. A recent report illustrates the difficulty in using TLR4 antagonism to reduce drinking behavior (McCarthy et al. 2017). TLR4 antagonism does, however, reduce conditioned place preference for ethanol (Jacobsen et al. 2018). Clearly, these interactions are complex, but they will likely produce novel therapeutic targets. Additionally, the interaction of stress and immune activation needs further investigation. Stress is a key feature in the cycle of addiction, however addiction-related stress is probably quite different from other non-addiction-related stressors, and is difficult to model in rodents. Neuroimmune activation causes both cognitive and emotive effects, leading to dysfunction (Dantzer et al. 2008; Yirmiya and Goshen 2011; Okun et al. 2010; Hanke and Kielian 2011). These mechanisms might contribute to the progressive and persistent nature of addiction. This work suggests that innate immune activation and TLR signaling are essential for ethanol-induced pathology. Though much of this work is convincing, important gaps remain regarding the precise mechanisms that cause

immune induction and the precise impact of neuroimmune activation. Nonetheless, sufficient findings are present to warrant the investigation of neuroimmune therapies for the treatment or prevention of alcoholism.

3 Novel Immune Therapeutic Strategies for Addiction

3.1 Toward Novel Addiction Treatments Strategies Based on Immune Pharmacology

Several potential and tested neuroimmune therapies are presented in Table 3, some of which in clinical trials for alcohol use disorders. A challenge in interpreting rodent studies is the difficulty in translating animal drinking models to the human condition. Also, certain interventions may be more effective at different stages of addiction. Additionally, it will likely be important to consider at which point in the disease progression a therapeutic would be effective. For instance, immune therapies would likely not be of benefit in brain regions where permanent changes such as neurodegeneration have occurred. However, given the acute influences of cytokines on plasticity, neuroimmune therapies might also aid in the recovery of normal synaptic function in other regions. Below, we list several potential neuroimmune treatment strategies (Table 3). There are already several FDA approved drugs, which have immune modulating effects in the brain. Minocycline, for example, is a tetracycline antibiotic that also regulates microglial function (Plane et al. 2010) and reduces ethanol self-administration (Agrawal et al. 2011; Qin and Crews 2012b) in vivo. Phosphodiesterase 4 (PDE4) inhibitors exert anti-inflammatory actions via NF-κB inhibition presumably through a cAMP-mediated mechanism (Jimenez et al. 2001), and have also been found to reduce ethanol self-administration in vivo (Blednov et al. 2014; Bell et al. 2015; Hu et al. 2011). Previously, ibudilast was found to reduce some of the rewarding effects of methamphetamine in a placebo-controlled trial (Worley et al. 2016) and may have efficacy in alcoholism. Currently, a phase I clinical trial with the PDE4 inhibitor ibudilast is underway for alcoholism. PPARγ agonists such as pioglitazone can act as microglial inhibitors and may be helpful in alcohol use disorders (Storer et al. 2005). Pioglitazone, for example, is a PPARγ agonist that has been shown to reduce neurotoxicity in models of fetal alcohol spectrum disorder (Kane et al. 2011; Drew et al. 2015). These drugs, and other, that have been shown to be effective in rodents should be considered for clinical therapeutic investigations. As the basic understanding of the neuroimmune contributions to the various stages of addiction increases, more targeted and strategic neuroimmune therapies will be developed.

3.2 Conclusions

In conclusion, the neuroimmune contributions to the pathology of alcoholism is a new and exciting field. This work suggests that innate immune activation and TLR

Table 3 Potential neuroimmune therapies for the treatment of addiction

Drug	Mechanism		CNS activity
	Primary	Immune	
Minocycline	Tetracycline antibiotic	Microglial inhibitor	Reduces alcohol self-administration (free-choice voluntary drinking) (Agrawal et al. 2011) Reduces ethanol microglia activation (Qin and Crews 2012b) Prevents reinstatement of morphine and amphetamine seeking (Arezoomandan and Haghparast 2016; Attarzadeh-Yazdi et al. 2014)
Rapamycin	Macrolide antibiotic	mTORC1 inhibitor	Reduces binge ethanol intake in male mice (Cozzoli et al. 2016) Neuroprotection via autophagy promotion (Chen et al. 2012; Pla et al. 2016)
Azithromycin	Macrolide antibiotic	Microglial inhibitor	Promotes anti-inflammatory M2 microglial activation state (Zhang et al. 2015)
Rifampin	Bacterial RNA polymerase inhibitor	TLR4 inhibition	Inhibits microglia activation to TLR4 (Bi et al. 2011; Wang et al. 2013)
Indomethacin	COX-2 inhibitor		Reduces ethanol self-administration in Sprague-Dawley rats (George 1989) Reduces ethanol neurotoxicity in cortex, hippocampus, and cerebellum (Pascual et al. 2007)
Simvastatin	HMG-CoA reductase inhibitor	NF-κB inhibition	Reduces inflammation and neurotoxicity to ischemia and traumatic brain injury in rodents (Sironi et al. 2006; Lim et al. 2017)
Glycyrrhizin	HMGB1 inhibition		Blocks ethanol-induced cytokine release in hippocampal slice culture (Zou and Crews 2014) Reduces neuroinflammation after traumatic brain injury in rodents (Okuma et al. 2014)
Pioglitazone, DHA	PPARγ agonists		Reduces toxicity and pro-inflammatory cytokines in a rodent fetal alcohol spectrum disorder model (Kane et al. 2011; Drew et al. 2015)
Ibudilast, mesopram, rolipram, CDP 840	Phosphodiesterase 4 inhibition		Reduces ethanol intake in two-bottle choice in C57BL/6J mice (Blednov et al. 2014) Reduces ethanol self-administration in p-rats and dependent mice (Bell et al. 2015)

(continued)

Table 3 (continued)

Drug	Mechanism		CNS activity
	Primary	Immune	
Naltrexone/ naloxone, nalmefene, and GSK1521498	μ-opioid antagonists	TLR4 inhibition	Reduces alcohol self-administration in rodents (Ripley et al. 2015) Binds TLR4 adaptor protein MD2 (Hutchinson et al. 2010; Wang et al. 2016) Prevents neuroimmune activation by ethanol (Montesinos et al. 2017)
Etanercept	TNFα antagonist		Prevents REM sleep disruption in alcoholics (Irwin et al. 2009)
Amlexanox	IKK/TBK1 inhibitor		Reduces ethanol self-administration (McCarthy et al. 2017)

signaling are essential for ethanol-induced pathology. Much of this work is convincing, however, critical gaps remain regarding the underlying mechanisms leading to immune induction and the precise impact of neuroimmune activation in the stages of addiction. Also, work is needed to identify particular circuits that may be more susceptible to deleterious effects of neuroimmune activation. Nonetheless, this field opens the possibility for new therapeutic interventions for alcoholism that could be efficacious at different stages of the disease. Nonetheless, sufficient findings are present to warrant the investigation of neuroimmune therapies for the treatment or prevention of alcoholism.

Acknowledgments We thank the National Institute on Alcohol Abuse and Alcoholism for its support through the Neurobiology of Adolescent Drinking in Adulthood (NADIA) consortium (AA020024, AA020023), the Bowles Center for Alcohol Studies (AA011605), the U54 collaborative partnership between NCCU and UNC (AA019767), the K08 award program (AA024829), and the Monkey Alcohol and Tissue Research Resource (MATRR-R24 AA019431).

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Ethanol and Cytokines in the Central Nervous System

Marisa Roberto, Reesha R. Patel, and Michal Bajo

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Abstract

The innate immune system plays a critical role in the ethanol-induced neuroimmune response in the brain. Ethanol initiates the innate immune response via activation of the innate immune receptors Toll-like receptors (TLRs, e.g., TLR4, TLR3, TLR7) and NOD-like receptors (inflammasome NLRs) leading to a release of a plethora of chemokines and cytokines and development of the innate immune response. Cytokines and chemokines can have pro- or anti-inflammatory properties through which they regulate the immune response. In this chapter, we will focus on key cytokines (e.g., IL-1, IL-6, TNF-α) and chemokines (e.g., MCP-1/CCL2) that

M. Roberto (✉) · R.R. Patel · M. Bajo
Department of Neuroscience, The Scripps Research Institute, La Jolla, CA, USA
e-mail: mroberto@scripps.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_77

mediate the ethanol-induced neuroimmune responses. In this regard, we will use IL-1β, as an example cytokine, to discuss the neuromodulatory properties of cytokines on cellular properties and synaptic transmission. We will discuss their involvement through a set of evidence: (1) changes in gene and protein expression following ethanol exposure, (2) association of gene polymorphisms (humans) and alterations in gene expression (animal models) with increased alcohol intake, and (3) modulation of alcohol-related behaviors by transgenic or pharmacological manipulations of chemokine and cytokine systems. Over the last years, our understanding of the molecular mechanisms mediating cytokine- and chemokine-dependent regulation of immune responses has advanced tremendously, and we review evidence pointing to cytokines and chemokines serving as neuromodulators and regulators of neurotransmission.

Keywords
Alcohol · Inflammatory mediators · Neuroimmune system · Synaptic transmission

1 Introduction

Innate immunity is the first line of defense against an immune challenge (e.g., infection, toxin, and trauma), and the response is characterized by limited specificity and a lack of memory. Regardless of the type of stimulus, the neuroimmune response involves activation of receptors of the innate immune system and release of inflammatory mediators. Inflammatory mediators comprise a heterogeneous group of factors, including cytokines, prostaglandins, free radicals, complement system, acute phase proteins, and neurotransmitters. These mediators regulate diverse aspects of the immune response including its intensity and duration. In general, the immune response/inflammation encompasses innate and adaptive immune responses that work together through direct cell contacts and through interactions involving chemical mediators (e.g., cytokines, antibodies). Contrary to innate immunity, the adaptive immune response is very specific, develops slowly, and shows memory (repeated challenge with the same microbe induces a faster and stronger response) (Abbas et al. 2018; Lydyard et al. 2011). This chapter focuses on a group of inflammatory mediators – cytokines and chemokines – and their role in the ethanol-induced neuroimmune response and adaptive changes in the brain.

1.1 Cytokines

Cytokines are a group of more than 300 soluble glycoproteins that are produced by cells in response to immunological stimuli (microbes, toxins, tissue damage, etc.). Cytokines are characterized by pleiotropic, redundant, synergistic, and antagonistic effects and play a crucial role in regulation of the innate and adaptive immune response. The term "cytokine" encompasses several classes of proteins including interleukins, chemokines, tumor necrosis factor, interferons, and growth factors (Abbas et al. 2018; Lydyard et al. 2011). Members from each of these cytokine

subgroups are involved in ethanol-induced pathology in the central nervous system (CNS) (Montesinos et al. 2016; Crews et al. 2017).

Interleukins (ILs) mediate signaling between cells of the immune system. ILs are produced by a variety of cells and are involved in regulation of cell growth, differentiation, and motility of immune cells (Vosshenrich and Di Santo 2002). Chemokines, such as MCP-1/CCL2 (monocyte chemotactic protein 1/chemokine ligand 2), are involved in leukocyte trafficking under both homeostatic and inflammatory conditions (Bachelerie et al. 2014). The tumor necrosis factor (TNF) family are characterized by their critical role in the inflammatory responses as well as in homeostatic processes (Sedger and McDermott 2014; Probert 2015). Interferons (IFNs) are pro-inflammatory molecules that are essential for innate and adaptive immunity and provide critical protection during early stages of viral, bacterial, or pathogen infections (Nallar and Kalvakolanu 2014). Growth factors include members of colony-stimulating factors (CSF) which mediate development, differentiation, and expansion of cells of the myeloid series and transforming growth factor β (TGFβ) which inhibits activation of macrophages and growth of B and T cells and is cytotoxic (Abbas et al. 2018; Lydyard et al. 2011).

The diversity of cytokine molecules reflects their broad functional roles in regulation of immune responses and homeostatic processes in peripheral organs as well as in the CNS. During the immune response, cytokines act in concert via complex interactions to regulate gene expression, cytokine release, and induction and termination of cytokine activity. While these interactions are fine-tuned under physiological conditions, their imbalance often leads to the development of pathological immune responses that are associated with numerous disorders.

2 Cytokines in the CNS

In the CNS, cytokines are produced locally, primarily by glial cells, but all CNS cell types are capable of synthesizing cytokines (Becher et al. 2017). Under physiological conditions, some cytokines are produced constitutively at relatively low levels. However, cytokine levels are significantly increased after various CNS and PNS (peripheral nervous system) injuries, seizures, or infections (Vezzani and Viviani 2015). While activation of cytokine signaling in glial cells is crucial for the immune response (Allan et al. 2005; Vezzani et al. 2011), cytokine signaling in neurons induces rapid and often persistent changes in excitability and/or presynaptic neurotransmitter release (Vezzani and Viviani 2015). In addition to the local production of cytokines, cytokines are also transported across the blood-brain barrier (BBB) from the periphery via active and passive transporter systems (Erickson et al. 2012; Erickson and Banks 2011; Banks 2015) and are produced by CNS-invading leukocytes (Callahan and Ransohoff 2004). Activation of cytokine signaling in endothelial cells of the BBB mediates the recruitment of circulating leukocytes and in some cases induces breakdown of tight junctions resulting in a leaky, permeable BBB (Annunziata et al. 2002; Tsao et al. 2001; Pan and Kastin 2001; Librizzi et al. 2012; Vezzani and Friedman 2011; Rochfort and Cummins 2015). Additionally,

peripheral cytokines can communicate with the CNS by acting on vagal afferent inputs, which modulate cholinergic signaling in the brain (Hosoi et al. 2002a; Maier et al. 1998).

Beyond regulating the immune response, cytokines in the CNS are also involved in regulation of homeostasis of the nervous system (Becher et al. 2017). Cytokines play a critical role in synaptic pruning during development, synapse removal, neurogenesis, and modulation of synaptic transmission in the brain (Vezzani and Viviani 2015; Boulanger 2009; Kohman and Rhodes 2013; Levin and Godukhin 2017; Pribiag and Stellwagen 2014; Williamson and Bilbo 2013; Marin and Kipnis 2013). Thus, dysregulation of cytokines, for instance, by ethanol exposure, has a complex impact on brain physiology and can cause long-lasting neuroadaptive changes (Crews et al. 2017). The overall effect of cytokines on neurons and glia is dependent on several factors including interaction with other cytokines, age, sex/gender, brain region, type of stimulus, and previous history of immune challenges (Barker et al. 2011; Lobo-Silva et al. 2016; Bardou et al. 2014; Pascual et al. 2017; Knapp et al. 2016; Biswas and Lopez-Collazo 2009; Marshall et al. 2016a; Topper et al. 2015).

3 Neuroimmune System and Alcohol Use Disorders

The neuroimmune system and ethanol have a complex reciprocal interaction, wherein the neuroimmune system modulates the effects of ethanol on synaptic transmission, ethanol drinking, and alcohol-related behaviors and ethanol modulates the activity of the neuroimmune system. There are several lines of evidence supporting this bidirectional interaction. Genetic predisposition to increased ethanol/alcohol drinking is associated with polymorphisms in neuroimmune genes and altered gene expression of cytokines in humans (Pastor et al. 2005; Marcos et al. 2008; Sery et al. 2003) and rodents (Mulligan et al. 2006; June et al. 2015). Moreover, transgenic and pharmacological manipulation of cytokine signaling alters ethanol drinking, ethanol-related behaviors, and the molecular and cellular effects of ethanol in the CNS (June et al. 2015; Bajo et al. 2014, 2015a; Blednov et al. 2005, 2011, 2012, 2015a; Wu et al. 2011; Marshall et al. 2017; Lippai et al. 2013a). Reciprocally, ethanol exposure induces acute and chronic changes in brain cytokine production, making these interactions very complex. The severity and duration of the neuroimmune response represented by a particular cytokine profile vary with the type of ethanol exposure/drinking (e.g., binge consumption). Table 1 summarizes the acute and chronic ethanol-induced dysregulation of cytokine production in animal models and humans. Alcohol use disorder (AUD) is associated with a chronic neuroimmune response and persistently altered neuroimmune gene expression (Crews and Vetreno 2014). Human and animal studies suggest that key mediators of the ethanol-induced neuroimmune response and neuroadaptive changes in the CNS include interleukins IL-1β, IL-6, IL-10, chemokine MCP-1/CCL2, and TNF-α. This chapter will highlight our current understanding of the role of these cytokines in AUDs.

Table 1 Ethanol-induced changes in the brain cytokine gene expression and protein levels in the animal models and humans

Cytokine	Ethanol treatment	Brain region	Δ mRNA levels	Δ Protein levels
Animal models (rodents)				
IL-1β	Acute	Whole brain	= (Rajayer et al. 2013; Qin et al. 2008; Doremus-Fitzwater et al. 2014) ↓ (Doremus-Fitzwater et al. 2014)	↑ (Rajayer et al. 2013) = (Qin et al. 2008) ↓ (Doremus-Fitzwater et al. 2014)
		Cortex	↑ (Ahlers et al. 2015) = (Pascual et al. 2017; Ahlers et al. 2015; Whitman et al. 2013; Teng and Molina 2014)	= (Whitman et al. 2013; Gottesfeld et al. 2002)
		Hypothalamus (PVN)	↑ (Doremus-Fitzwater et al. 2014) = (Gano et al. 2017; Doremus-Fitzwater et al. 2014, 2015) ↓ (Doremus-Fitzwater et al. 2015)	= (Gottesfeld et al. 2002)
		Hippocampus	↑ (Doremus-Fitzwater et al. 2014) = (Gano et al. 2017; Doremus-Fitzwater et al. 2014, 2015) ↓ (Doremus-Fitzwater et al. 2015)	= (Gottesfeld et al. 2002)
		Cerebellum	= (Doremus-Fitzwater et al. 2014)	
		Amygdala	= (Gano et al. 2017; Doremus-Fitzwater et al. 2015)	
	Prolonged/binge	Whole brain	= (Qin and Crews 2014; Qin et al. 2008)	= (Qin et al. 2008; Qin and Crews 2014)
		Cortex	↑ (Pascual et al. 2017) = (Drew et al. 2015)	↑ (Montesinos et al. 2017; Tiwari and Chopra 2012, 2013)
		Hippocampus	↑ (Drew et al. 2015)	↑ (Tiwari and Chopra 2013)
		Cerebellum	↑ (Topper et al. 2015; Drew et al. 2015)	
		Striatum/NAc		↑ (Montesinos et al. 2017)
	Chronic	Cortex	↑ (Whitman et al. 2013; Alfonso-Loeches et al. 2013) = (Whitman et al. 2013) ↓ (Vetreno et al. 2013)	↑ (Alfonso-Loeches et al. 2013; Schneider et al. 2017; Diaz et al. 2016) = (Whitman et al. 2013; Alfonso-Loeches et al. 2013)
		Hypothalamus (PVN)	↓ (Doremus-Fitzwater et al. 2014)	

(continued)

Table 1 (continued)

Cytokine	Ethanol treatment	Brain region	Δ mRNA levels	Δ Protein levels
		Hippocampus	= (Doremus-Fitzwater et al. 2014)	↑ (Diaz et al. 2016; Zhu et al. 2007)
		Cerebellum	↑ (Lippai et al. 2013a)	↑ (Lippai et al. 2013a)
		Amygdala	↓ (Doremus-Fitzwater et al. 2014)	
		Striatum/NAc		↑ (Pascual et al. 2015)
IL-6	Acute	Cortex	= (Teng and Molina 2014)	= (Teng and Molina 2014)
		Hypothalamus (PVN)	↑ (Doremus-Fitzwater et al. 2014, 2015) = (Gano et al. 2017; Doremus-Fitzwater et al. 2014)	
		Hippocampus	↑ (Doremus-Fitzwater et al. 2014, 2015) = (Gano et al. 2017; Doremus-Fitzwater et al. 2014, 2015)	
		Cerebellum	↑ (Doremus-Fitzwater et al. 2014) = (Doremus-Fitzwater et al. 2014) ↓ (Doremus-Fitzwater et al. 2014)	
		Amygdala	↑ (Doremus-Fitzwater et al. 2015) = (Gano et al. 2017)	
	Prolonged/binge	Whole brain	↑ (Qin and Crews 2014)	↑ (Qin and Crews 2014)
		Cortex	= (Kane et al. 2013, 2014)	↑ (Marshall et al. 2013) = (Marshall et al. 2013)
		Hypothalamus (PVN)		↑ (Zhu et al. 2007)
		Hippocampus	= (Kane et al. 2013, 2014)	= (Marshall et al. 2013)
		Cerebellum	= (Kane et al. 2013)	
	Chronic	Cortex		↑ (Schneider et al. 2017)
		Hypothalamus (PVN)	= (Doremus-Fitzwater et al. 2014)	↑ (Emanuele et al. 2005)
		Hippocampus	= (Doremus-Fitzwater et al. 2014)	↑ (Schneider et al. 2017)
		Amygdala	↑ (Doremus-Fitzwater et al. 2014)	

IL-10	Acute	Whole brain	= (Qin et al. 2008)	
		Hippocampus		↑ (Suryanarayanan et al. 2016)
	Prolonged/binge	Whole brain	= (Qin et al. 2008)	↓ (Qin et al. 2008)
		Cortex		= (Marshall et al. 2013, 2016a)
		Hippocampus		↑ (Marshall et al. 2013) = (Marshall et al. 2013, 2016a)
		Cerebellum	= (Topper et al. 2015)	
	Chronic	Cortex		↓ (Schneider et al. 2017)
		Hippocampus		↓ (Schneider et al. 2017)
IL-1Ra	Chronic	Cerebellum		↑ (Lippai et al. 2013a)
TNF-α	Acute	Whole brain	↑ (Rajayer et al. 2013; Qin et al. 2008)	↑ (Rajayer et al. 2013) = (Qin et al. 2008)
		Cortex	↑ (Ahlers et al. 2015; Teng and Molina 2014) = (Ahlers et al. 2015; Whitman et al. 2013; Teng and Molina 2014)	↑ (Whitman et al. 2013; Amin et al. 2016) = (Gottesfeld et al. 2002)
		Hypothalamus (PVN)	↑ (Doremus-Fitzwater et al. 2014) = (Gano et al. 2017; Doremus-Fitzwater et al. 2014) ↓ (Doremus-Fitzwater et al. 2014, 2015)	
		Hippocampus	↑ (Doremus-Fitzwater et al. 2014) = (Gano et al. 2017; Doremus-Fitzwater et al. 2014) ↓ (Doremus-Fitzwater et al. 2014, 2015)	= (Gottesfeld et al. 2002)
		Cerebellum	= (Doremus-Fitzwater et al. 2014) ↓ (Doremus-Fitzwater et al. 2014)	
		Amygdala	= (Gano et al. 2017; Doremus-Fitzwater et al. 2015) ↓ (Doremus-Fitzwater et al. 2015)	
	Prolonged/binge	Whole brain	↑ (Qin et al. 2008; Qin and Crews 2014) = (Qin et al. 2008)	↑ (Qin et al. 2008; Qin and Crews 2014) = (Qin et al. 2008)

(continued)

Table 1 (continued)

Cytokine	Ethanol treatment	Brain region	Δ mRNA levels	Δ Protein levels
		Cortex	↑ = (Kane et al. 2013, 2014; Drew et al. 2015)	↑ (Pascual et al. 2017; Tiwari and Chopra 2012, 2013) = (Marshall et al. 2013, 2016a)
		Hypothalamus (PVN)		↑ (Zhu et al. 2007) = (Zahr et al. 2010)
		Hippocampus	↑ (Drew et al. 2015) = (Kane et al. 2013, 2014)	↑ (Marshall et al. 2016a; Tiwari and Chopra 2012, 2013) = (Marshall et al. 2013, 2016a; McClain et al. 2011)
		Cerebellum	↑ (Topper et al. 2015; Drew et al. 2015) = (Topper et al. 2015; Kane et al. 2013)	
		Striatum/NAc		↑ (Pascual et al. 2017)
	Chronic	Cortex	↑ (Vetreno et al. 2013; Whitman et al. 2013; Alfonso-Loeches et al. 2013) = (Whitman et al. 2013)	↑ (Alfonso-Loeches et al. 2013; Schneider et al. 2017; Diaz et al. 2016) = (Whitman et al. 2013)
		Hypothalamus (PVN)	= (Doremus-Fitzwater et al. 2014)	↑ (Emanuele et al. 2005)
		Hippocampus	↓ (Doremus-Fitzwater et al. 2014)	↑ (Schneider et al. 2017; Diaz et al. 2016)
		Cerebellum	↑ (Lippai et al. 2013a)	↑ (Lippai et al. 2013a)↓
		Amygdala	= (Doremus-Fitzwater et al. 2014)	
		Striatum/NAc		↑ (Pascual et al. 2015) = (Pascual et al. 2015)
MCP-1/ CCL2	Acute	Whole brain	↑ (Qin et al. 2008)	↑ (Roberson et al. 2011) = (Qin et al. 2008; Roberson et al. 2011)
		Cortex	↑ (Teng and Molina 2014) = (Pascual et al. 2017) (Kane et al. 2013; Whitman et al. 2013; Teng and Molina 2014)	↑ (Montesinos et al. 2017) = (Whitman et al. 2013)
		Hippocampus	↑ (Kane et al. 2013)	
		Cerebellum	↑ (Kane et al. 2013)	

	Prolonged/binge	Whole brain	↑ (Qin et al. 2008; Qin and Crews 2014)	↑ (Qin et al. 2008; Qin and Crews 2014) = (Qin et al. 2008)
		Cortex	↑ (Pascual et al. 2017; Kane et al. 2014; Drew et al. 2015) = (Kane et al. 2014)	↑ (Kane et al. 2014)
		Hippocampus	↑ (Kane et al. 2014; Drew et al. 2015) = (Kane et al. 2014) ↓	↑ (Kane et al. 2014)
		Cerebellum	↑ (Drew et al. 2015)	
		Striatum/NAc		↑ (Montesinos et al. 2017)
	Chronic	Cortex	↑ (Vetreno et al. 2013; Whitman et al. 2013) = (Whitman et al. 2013) ↓	↑ = (Whitman et al. 2013) ↓
		Cerebellum	↑ (Lippai et al. 2013a)	↑ (Lippai et al. 2013a)
		Striatum/NAc		↑ (Pascual et al. 2015)
Humans				
IL-1β	Alcoholics – acutely exposed to EtOH	CF		= (Umhau et al. 2014)
	Alcoholics (postmortem)	Hippocampus		↑ (Zou and Crews 2012)
IL-6	Alcoholics + hepatic encephalopathy	Superior frontal gyrus		= (Dennis et al. 2014)
		Precentral gyrus		= (Dennis et al. 2014)
IL-10	Alcoholics + hepatic encephalopathy	Superior frontal gyrus		= (Dennis et al. 2014)
		Precentral gyrus		= (Dennis et al. 2014)
TNF-α	Alcoholics – acutely exposed to EtOH	CF		= (Umhau et al. 2014) ↓ (Umhau et al. 2014)

(continued)

Table 1 (continued)

Cytokine	Ethanol treatment	Brain region	Δ mRNA levels	Δ Protein levels
MCP-1/ CCL2	Alcoholics (postmortem)	VTA		↑ (He and Crews 2008)
		Substantia nigra		↑ (He and Crews 2008)
		Hippocampus		↑ (He and Crews 2008)
		Amygdala		↑ (He and Crews 2008)
	Alcoholics – acutely exposed to EtOH	CF		↑ (Umhau et al. 2014)

We define "acute treatment" as a single administration or continuous application for less than 24 h. The "prolonged/binge treatment" includes several binge models and repeated ethanol exposure for less than 2 weeks, and "chronic treatment" corresponds to ethanol treatments exceeding 2 weeks. The primary reasons for the discrepancies in the direction of the ethanol effects on a particular cytokine among and within the studies include age (e.g., adolescent vs adult), sex, ethanol treatment/administration (e.g., intraperitoneal vs intragastric application or continuous vs intermittent treatment), and posttreatment time of the tissue collection (e.g., 1 day vs 28 days). The term "cortex" encompasses findings from the studies on the neocortex, frontal cortex, mPFC, and entorhinal and temporal cortex. We mark the direction of the ethanol effects on the cytokines (Δ mRNA/protein levels) as ↑ increase, = no change, and ↓ decrease in the mRNA or protein levels

CF cerebrospinal fluid, *VTA* ventral tegmental area, *PVN* paraventricular nucleus of the hypothalamus, *NAc* nucleus accumbens

4 The Interleukin-1 Family

The interleukin-1 (IL-1) family is a group of 11 cytokines that initiate and regulate inflammatory responses (Dinarello 2011). IL-1α/β and its cognate IL-1 receptor type 1 (IL-1R1) are expressed throughout the brain (Parker et al. 2000; French et al. 1999; Ericsson et al. 1995; Heida and Pittman 2005; Johnson et al. 2004; Hosoi et al. 2002b; Gayle et al. 1999; Cartmell et al. 1999; Taishi et al. 1997; Quan et al. 1998; Quan et al. 1996; Hagan et al. 1993) and are synthesized in both neurons (Allan et al. 2005) and glial cells (Blanco et al. 2005; Blanco and Guerri 2007). Specifically, IL-1R1 is enriched in postsynaptic compartments in rat hippocampus and cortex (Gardoni et al. 2011; Viviani et al. 2014). In general, IL-1α is produced constitutively, whereas IL-1β synthesis is induced and requires activation of the inflammasome pathway. The inflammasome is a multiprotein complex mainly functioning as a platform for the activation of inflammatory caspases to produce pro-inflammatory cytokines (IL-1β and IL-18) and as a trigger for the release of proteins involved in coordination of cell proliferation and tissue repair. First, an initial immune stimulus induces gene expression and protein synthesis of the inactive proIL-1β. The release of an active IL-1β requires a second stimulus that activates the inflammasome, which leads to cleavage of the proIL-1β by caspase 1 (Keyel 2014; Lamkanfi and Dixit 2014). Notably, activation of the inflammasome pathway, particularly NLRP3/ASC inflammasome, plays a critical role in regulation of the alcohol-induced neuroimmune response (Lippai et al. 2013a; Zou and Crews 2012; Wang et al. 2015; Alfonso-Loeches et al. 2014; Alfonso-Loeches et al. 2015).

The pro-inflammatory activities of IL-1α and IL-1β are mediated by downstream signaling of IL-1R1. IL-1α/β binds to the extracellular domain of IL-1R1 leading to the recruitment of accessory proteins (e.g., the co-receptor IL-1R1 accessory protein (IL-1RAcP)), formation of a receptor heterodimeric complex (comprised of IL-1α/β, IL-1R1, and IL-1RAcP), and assemblage with the intracellular adaptor protein MyD88. Downstream of IL-1R1, many intracellular signaling pathways are activated such as NF-κB, c-Jun N-terminal kinase, and p38 MAPK. Additionally, transcription factors, which induce gene expression of the inflammatory mediators including IL-1α/β, are also activated (Cohen 2014). Importantly, IL-1α/β-IL-1R1 signaling is regulated by an endogenous antagonist (IL-1Ra) and a decoy receptor (IL-1R2). IL-1Ra (IL-1 receptor antagonist) competes with IL-1α/β for binding sites on IL-1R1, and IL-1α/β binds to the decoy receptor IL-1R2, which does not assemble into the IL-1R1/IL-1RAcp/MyD88 complex (Garlanda et al. 2013; Krumm et al. 2014). In both cases, the inhibition of IL-1α/β-mediated response is carried out by preventing the activation of downstream IL-1R1 signaling (Garlanda et al. 2013).

Based on the available literature, ethanol does not induce changes in the IL-1α levels in the brain (Lippai et al. 2013a). While this does not exclude the possibility of IL-1α playing a role in the ethanol-induced neuroimmune response, IL-1α does not appear to play a critical role in ethanol effects in the CNS (Bajo et al. 2015a, b). Here, we will focus on IL-1β which play a critical role in the ethanol-induced neuroimmune response in the CNS (Szabo and Lippai 2014).

The mechanisms by which IL-1β exerts its effects can be broadly categorized into two branches: (1) primarily immune cell-mediated effects and (2) direct neuronal effects. The first branch of the IL-1β mechanisms encompasses various processes of the neuroimmune response (e.g., free radical generation, activation of glial cells) in which the IL-1β serves as a key regulator. The second branch includes the direct regulation of homeostasis in the CNS by IL-1β and IL-1β − dependent modulation of synaptic transmission. Importantly, the individual mechanisms from both branches do not act independently, but rather work in parallel, influencing the actions of each other.

The IL-1β system modulates the functional activity of neurons in a cell- and brain region-specific manner including excitability, neurotransmitter receptors, neurotransmitter release, and synaptic plasticity. For example, IL-1β directly modulates voltage-gated ion channels (Vezzani and Viviani 2015); it increases firing in Purkinje cells (Motoki et al. 2009), decreases firing in dorsal raphe nucleus serotonergic neurons (Brambilla et al. 2007; Manfridi et al. 2003), and has dual effects in orbitofrontal cortex neurons (Lukats et al. 2005). In the hippocampus (including hippocampal neuronal cell cultures), IL-1β increases the membrane expression of GABA (γ-aminobutyric acid) receptors (Serantes et al. 2006; Wang et al. 2012) and IL-1R1 at synaptic sites, where IL-1R1 colocalizes and binds to the GluR2B subunit of NMDA (N-methyl D-aspartic acid) receptors (Gardoni et al. 2011). IL-1β-IL-1R can increase NMDA receptor phosphorylation (e.g., GLuR2B subunit) leading to an increase in NMDA-mediated calcium (Ca^{2+}) flux, excitability, and excitotoxicity (Viviani et al. 2003). The dual effects of IL-1β on presynaptic GABA and glutamate release as well as postsynaptic inhibitory and excitatory activity are specific to neuronal type and brain region (Bajo et al. 2015b; Feleder et al. 1998; Miller et al. 1991; Mishra et al. 2012; Murray et al. 1997; Sama et al. 2008; Tabarean et al. 2006; Zeise et al. 1992, 1997). Moreover, IL-1β inhibits synaptic plasticity in CA1 and dentate gyrus neurons of the hippocampus (Zeise et al. 1992; Dunn et al. 1999; Lin et al. 2006; O'Connor and Coogan 1999; Rothwell and Luheshi 2000).

4.1 IL-1β and Alcohol

There are several lines of evidence supporting the critical role of IL-1β in the neuropathogenesis and behavioral changes associated with alcohol dependence. In humans, polymorphisms in *Il1rn* and *Il1b*, the genes encoding IL-1Ra and IL-1β, respectively, are associated with a susceptibility to alcoholism in Spanish men (Pastor et al. 2005). Similarly, mice with a genetic predisposition to high alcohol consumption show altered expression of several genes of the IL-1/IL-1R system, including *Ilf5*, *Ilf6*, *Ilf8*, *Irak4*, and *Il1rn*. All of these genes, except *Irak4*, are also located within QTLs (quantitative trait locus) for human alcoholism susceptibility and are considered candidate genes for alcohol drinking (Mulligan et al. 2006). ILf5, ILf6, and ILf8 are ligands for IL-1R2 (Towne et al. 2004). *Irak4* encodes the protein IRAK4 (IL-1 receptor-associated kinase 4), which plays a key role in the activation of NF-κB signaling (O'Neill 2008). Interestingly, high alcohol-preferring (HAP)

mice have altered levels of several genes involved in the NF-κB pathway (*Casp8*, *Fadd*, *Ikbkb*, *Ikbkg*, *Map3k1*, *Map3k7*, *Tradd*), through which IL-1α/β-IL-1R1 mediates its biological action (Mulligan et al. 2006). Follow-up behavioral studies show the involvement of some of these genes in alcohol drinking and preference. *Il1rn* encodes the IL-1Ra protein that is an endogenous competitive antagonist of IL-1R1. *Il1rn* knockout mice exhibit a reduction in alcohol drinking and preference (Blednov et al. 2012), increased ethanol clearance and decreased ethanol-induced conditioned taste aversion, increased sensitivity to the sedative/hypnotic effects of ethanol and flurazepam, and reduced severity of acute ethanol withdrawal. Pretreatment with exogenous IL-1Ra (Kineret) reverses some of the behavioral phenotypes of *Il1rn* KO mice; specifically it reduces the ethanol- and flurazepam-induced sedation and restores the severity of acute ethanol withdrawal (Blednov et al. 2015a). Mice lacking the *Il1r1* gene, encoding IL-1R1, exhibit the opposite phenotype of *Il1rn* KO mice – decreased ethanol-induced sedation and increased severity of ethanol withdrawal – indicating that IL-1R1 signaling plays a crucial role in these behaviors. However, the findings that ethanol intake and preference are not altered in *Il1r1* KO mice and recovery from ethanol-induced motor incoordination is only altered in female *Il1r1* KO mice suggest that these alcohol-related behaviors are not solely regulated by the IL-1β system (Blednov et al. 2015a). Moreover, systemic administration of IL-1Ra reduces alcohol-induced sedation and motor impairment recovery time in mice (Wu et al. 2011) and also prevents alcohol-induced neuroinflammation (Lippai et al. 2013a).

Pharmacological manipulation of the IL-1 system selectively in the CNS provides further evidence for a critical role of the brain IL-1 system in several alcohol-related behaviors. Intracerebroventricular administration of IL-1β increases alcohol withdrawal-induced anxiety (Breese et al. 2008), while bilateral infusion of IL-1Ra into the basolateral amygdala (BLA), but not the central nucleus of the amygdala (CeA), reduces ethanol consumption with no impact on either sucrose drinking or open-field locomotor activity, a behavioral measure of anxiety (Marshall et al. 2016b). Overall, these evidences indicate that IL-1β plays a critical role in activation of the ethanol-induced immune response in the brain and is involved in the regulation of critical neurocircuitries mediating the alcohol-related behaviors.

4.2 IL-1β Mechanisms of Action

Evidence for the involvement of IL-1β and its signaling pathways in alcohol-related behaviors are compelling. Indeed, ethanol increases IL-1β levels in neuronal and glial cell cultures (Zou and Crews 2012; Rajayer et al. 2013; Boyadjieva and Sarkar 2010; Lawrimore and Crews 2017) and in specific brain regions in animal models of AUDs as well as in humans (see Table 1). In this regard, the hippocampus, PFC, and cerebellum seem to be the most sensitive to ethanol-induced dysregulation of IL-1/IL-1R1 signaling (Lippai et al. 2013a, b; Qin et al. 2008; Valles et al. 2004). However, the mechanisms through which IL-1β modulates alcohol-related behaviors are still not fully understood. Therefore, the focus of current research has extended to

the other brain regions such as the amygdala, which plays a critical role in alcohol dependence and withdrawal (Koob and Volkow 2016). Thus, here, we will summarize our current understanding of the mechanisms of action of IL-1β and IL-1β-ethanol interactions at the cellular and behavioral levels in the CeA, BLA, and hippocampus.

4.3 IL-1β in the CeA

The CeA, a major component of the extended amygdala, is a primarily GABAergic nucleus involved in stress-, fear-, and anxiety-like behavior (Davis et al. 1994) and excessive drinking (Koob and Volkow 2016; Roberto et al. 2010). The GABAergic system tightly controls neuronal excitability (Klausberger and Somogyi 2008; Nuss 2015), and it is critical in the development of alcohol dependence (Holmes and Wellman 2009; Silveri 2014).

Modulation of GABA-A receptors alters many ethanol behaviors (Blednov et al. 2003, 2013; Boehm et al. 2004). Specifically, muscimol, a GABA-A receptor agonist, injection into the CeA greatly reduces ethanol self-administration, but only in dependent rats (Roberts et al. 1996), and a GABA-A antagonist reduces ethanol self-administration (Hyytia and Koob 1995) in nondependent rats. The CeA has abundant corticotrophin-releasing factor (CRF)-containing fibers and CRF receptors (Uryu et al. 1992) and is thought to be a target of the peripheral neuroimmune system (Konsman et al. 2008). CRF1 receptors play an essential role in ethanol's effects on GABA release in the CeA and in ethanol dependence (Roberto et al. 2003, 2010; Nie et al. 2004, 2009). Interestingly, facilitation of ethanol withdrawal-induced anxiety by TNFα or MCP-1/CCL2 microinjection into the CeA is dependent on CRF (Knapp et al. 2011), and CRF-amplified neuronal TLR4/MCP-1 signaling in the CeA regulates alcohol self-administration (June et al. 2015). Moreover, IL-1β and IL-1Ra regulate GABAergic transmission in the CeA (Bajo et al. 2015a, b). Under basal conditions, IL-1R is detected in the amygdala (Frost et al. 2001), but expression of IL-1β and IL-1Ra is not detectable but rather appears to be inducible in the CeA (Konsman et al. 2008; Eriksson et al. 2000), suggesting that modulation of basal GABAergic transmission with acute application IL-1Ra is likely through IL-1α, which is constitutively expressed. Systemic IL-1β and LPS administration activates the CeA, as indicated by an increase in gene expression of the immediate early gene product cFos (Konsman et al. 2008; Frost et al. 2001; Dayas et al. 2001). At the cellular level, IL-1β significantly decreases amplitudes of evoked inhibitory GABA-A-mediated postsynaptic potentials (eIPSP), without affecting paired-pulse facilitation (PPF), a paradigm to assess pre- and postsynaptic mechanisms for evoked responses. Interestingly, IL-1β has dual effects on action potential-independent miniature inhibitory postsynaptic currents (mIPSCs) in CeA neurons: in the majority of cells, IL-1β increases mIPSC frequency suggesting an increase in presynaptic vesicular GABA release. However, in some CeA neurons, IL-1β decreases vesicular GABA release as well as postsynaptic GABA-A receptor function represented by a decrease in mIPSC

amplitude. Consistent with the IL-1β effects, IL-1Ra alone had dual effects on mIPSCs, and it also blocks the effects of IL-1β on CeA GABA transmission (Bajo et al. 2015a, b).

Further, acute ethanol facilitates GABA transmission in the CeA (Roberto et al. 2003). IL-1β interacts with the effects of acute ethanol on GABA transmission in the CeA. Although IL-1β pretreatment does not block the ethanol-induced facilitation of evoked responses, IL-1β occludes ethanol's effects on presynaptic vesicular GABA release in CeA neurons responding to IL-1β. Overall, these findings indicate that the IL-1 system is involved in tonic regulation of GABA transmission and that IL-1β interacts with the ethanol-induced enhancement of GABAergic transmission in the CeA (Bajo et al. 2015b).

The endogenous IL-1Ra is an anti-inflammatory element that may play a critical role in the development of alcohol dependence (Mulligan et al. 2006; Blednov et al. 2012; Wu et al. 2011; Lippai et al. 2013a). Transgenic mice lacking endogenous IL-1Ra (*Il1rn* KO) exhibit reduced alcohol intake (Blednov et al. 2012) and prolonged loss of the righting reflex (LORR) induced by ethanol or by flurazepam, a positive allosteric modulator of the GABA-A receptor (Blednov et al. 2015a). Also, GABAergic neurotransmission in the CeA of *Il1rn* KO mice is disrupted. Notably, both baseline-evoked GABA responses and baseline frequency of action potential-dependent spontaneous inhibitory postsynaptic currents (sIPSCs), but not mIPSCs, are significantly increased in these KO mice compared to wild-type (WT) mice, indicating increased GABA release in the CeA of KO mice. Acute application of ethanol increases the frequency of sIPSCs and mIPSCs in the vast majority of WT CeA neurons, but these effects are observed only in about half of the KO CeA neurons. Pretreatment with exogenous IL-1Ra (Kineret) reverses this increase in KO mice without altering the frequency in WT mice. Kineret is also capable of restoring the ethanol-induced increase in GABA release in KO mice, indicating that some of the cellular phenotypes in *Il1rn* KO mice are rescued by application of exogenous IL-1Ra (Bajo et al. 2015a). This suggests that IL-1R1 antagonism regulates basal GABA release and plays a key role in the effects of ethanol at inhibitory synapses in the CeA.

4.4 IL-1β in the BLA

Acute application of IL-1β hyperpolarizes the membrane and decreases input resistance in most BLA neurons. The hyperpolarization induced by IL-1β is dose-dependent, reversible, action potential independent, and blocked with a GABA-A antagonist. IL-1β inhibits excitatory and inhibitory responses evoked by stimulating either the bed nucleus of stria terminalis or the lateral amygdala via presynaptic mechanisms. Thus, IL-1β hyperpolarizes the membrane through indirect mechanisms, possibly by enhancing the action of endogenous GABA in the BLA, and inhibits excitatory and inhibitory transmission at presynaptic sites (Yu and Shinnick-Gallagher 1994).

Binge-like ethanol drinking induces a significant increase in IL-1β mRNA and protein expression within the amygdala, but not in the CeA. Interestingly, bilateral infusion of IL-1Ra into the BLA, but not the CeA, reduce ethanol drinking

without affecting sucrose drinking or open-field locomotor activity (Marshall et al. 2016b). These results highlight a specific role for IL-1 receptor signaling in the BLA in modulating binge-like ethanol consumption and indicate that pro-inflammatory cytokines can be induced prior to progression into alcohol dependence.

4.5 IL-1β in the Hippocampus

The hippocampus expresses a high density of IL-1β receptors presumably on dendrites of granule cells (Takao et al. 1990). Exogenously applied IL-1β enhances neuronal excitability and increases NMDA receptor function. Indeed, data from primary rat hippocampal neuron cultures suggests that IL-1β increases NMDA receptor function through activation of tyrosine kinases and subsequent NR2A/B subunit phosphorylation (Viviani et al. 2003). IL-1β reduces seizure thresholds and inhibition of IL-1R1 by its antagonist limits seizures (Vezzani et al. 1999). Moreover, convulsant and/or excitotoxic stimuli increase the production of IL-1β in microglia-like cells in the hippocampus (Vezzani et al. 1999). A later study clarified the mechanism of IL-1β-associated seizures and the interaction between IL-1β and Ca^{2+} mobilization on glutamate and GABA release using mice hippocampal mini-slice (Zhu et al. 2006). Both basal and K^+-evoked GABA release are regulated by Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} releasing system (CICR). Similarly, K^+-evoked glutamate release is also regulated by Ca^{2+} influx and CICR, but basal glutamate release is not. IL-1β increases basal release of glutamate and GABA depending on the activation of Ca^{2+} influx and ryanodine receptor (RyR)-sensitive CICR. During neuronal hyperexcitability, the effect of IL-1β on GABA release is predominantly modulated by Ca^{2+} influx and RyR-sensitive CICR (Zhu et al. 2006).

IL-1β can also impact neuronal plasticity. Low, physiological levels of IL-1β play a role in long-term potentiation (LTP), an important cellular correlate of learning and memory, while high, pathological levels can disrupt this process. Blockade of IL-1β signaling by its antagonist, IL-1Ra, impairs memory. However, addition of excessive IL-1β also impairs memory (Goshen et al. 2007). Therefore, immune signaling impacts plasticity through finely tuned changes in cytokine levels that alter neuronal activity, neural circuitry, and consequently behavioral phenotypes (Crews et al. 2017).

IL-1β can also affect neurogenesis, the process of generating functional neurons from neural precursors, in the hippocampus. Inflammation (Ryan and Nolan 2016) and chronic stress (Kreisel et al. 2014) reduce neurogenesis and cause depression-like behavior. In particular, stress induces IL-1β expression in the hippocampus, which decreases neurogenesis and contributes to depression. Blockade of IL-1β signaling inhibits stress-induced decreases in neurogenesis and depression-like behavior (Koo and Duman 2008). The increased gene expression and protein levels of IL-1β in the hippocampus following prolonged/binge and chronic ethanol exposure, found in animal models as well as in human alcoholics (Table 1), indicate that

IL-1β may play a critical role in the ethanol-reduced hippocampal neurogenesis (Geil et al. 2014). Indeed, blocking IL-1β or inflammasome signaling reverses the effects of ethanol on neurogenesis (Geil et al. 2014). These findings indicate that inflammasome and IL-1β mediate the ethanol-induced inhibition of the hippocampal neurogenesis (Zou and Crews 2012).

Thus, it is clear that the IL-1β system plays a neuromodulatory role and interacts with ethanol in CeA/BLA/hippocampus neurons. At the same time, there are still many unanswered questions regarding the mechanisms mediating brain region differences in the IL-1β effects and neuroadaptive changes of the IL-1β system induced by chronic ethanol exposure and withdrawal.

5 Interleukin-6

Interleukin-6 (IL-6) is a prototypical pro-inflammatory cytokine involved in the transition from innate to adaptive immunity. IL-6 plays a major role in the neuroimmune response to brain injury and is associated with multiple neurobiological (e.g., multiple sclerosis, Parkinson's disease, Alzheimer's disease) and psychiatric (major depression, post-traumatic stress disorder, substance use disorders) disorders (Erta et al. 2012). In addition to mediating the neuroimmune response, IL-6 is critical in neurogenesis and the regulation of various physiological processes (e.g., food intake, body weight, body temperature, stress, sleep-awake behavior, etc.) (Wallenius et al. 2002; Herrmann et al. 2003; Chai et al. 1996; Mastorakos et al. 1993; Morrow and Opp 2005; Dimitrov et al. 2006). Neurons, astrocytes, microglia, and endothelial cells are essential sources of IL-6, but astrocytes are the primary source of IL-6 under physiological conditions and during alcohol exposure in the CNS (Ye and Johnson 1999; Fattori et al. 1995; Choi et al. 2014; Farina et al. 2007). Production of IL-6 in brain cells is regulated by other cytokines and inflammatory factors (e.g., IL-1β and TNF-α) as well as by neurotransmitters and neuropeptides (e.g., norepinephrine, serotonin, substance P) (Erta et al. 2012; Norris and Benveniste 1993; Maimone et al. 1993; Lieb et al. 2005; Gitter et al. 1994). IL-6 signaling is initiated by binding of IL-6 to the IL-6 receptor (IL-6R) and recruitment of additional accessory proteins including gp130, which leads to the activation of major signaling pathways including JAK2/ STAT3, p44/42 MAPK, and PI3-K (Schaper and Rose-John 2015). IL-6 modulates gene expression of many inflammatory mediators and proteins involved in apoptosis and other processes (Erta et al. 2012). At the cellular level, IL-6 has an inhibitory effect on sodium (Na^+) and Ca^{2+} voltage-gated ion channels that may serve as a neuroprotective mechanism in the CNS (Vezzani and Viviani 2015; Li et al. 2014). Moreover, IL-6 modulates glutamate receptor (mGluR2/3) expression and glutamate-mediated excitotoxicity (Conroy et al. 2004; Orellana et al. 2005; Qiu et al. 1995). IL-6 also reversibly decreases GABA-A-mediated currents, likely via modulation of GABA-A receptor compartmentalization and PI3-K-Akt pathway (Garcia-Oscos et al. 2012). The direct effects of IL-6 on cellular physiology and synaptic

transmission indicate that dysregulation of IL-6 signaling may lead to a significant disturbance in network activity in a brain region-specific manner.

5.1 IL-6 and Alcohol

A polymorphism in *Il6*, the gene encoding IL-6, is associated with alcoholism in humans (Marcos et al. 2008; Sery et al. 2003), and genomic studies show modifications in *Il6* gene expression in alcohol-preferring rodents (Mulligan et al. 2006). Transgenic mice with a null mutant *Il6* have lower ethanol intake and ethanol preference compared to WT mice (Blednov et al. 2012). On the other hand, transgenic mice with elevated astrocyte production of IL-6 in the CNS (IL-6tg mice) show increased susceptibility to acute alcohol withdrawal hyperexcitability (Hernandez et al. 2016).

While ethanol has mixed effects on the IL-6 levels in neuronal and glial cell cultures (Boyadjieva and Sarkar 2010; Lawrimore and Crews 2017; Sarc et al. 2011; Chaturvedi et al. 2012; Wilhelm et al. 2016), both acute and chronic ethanol exposure increases IL-6 levels in a brain region- and ethanol exposure (time and dose)-specific manner (see Table 1). In addition to the direct effects of ethanol on IL-6 levels in the brain, ethanol's effects on IL-6 levels might also be under conditioned control. Repeated pairings between distinctive odor cues (conditional stimulus) and ethanol can result in elevation of IL-6 levels in the hippocampus and amygdala upon presentation of the odor cues alone (Gano et al. 2017). At the synaptic level, IL-6tg mice exhibit an altered response in hippocampal LTP to acute ethanol. While acute ethanol depresses fEPSPs (field excitatory postsynaptic potentials), PTP (post-tetanic potentiation), and LTP and does not affect sPS (secondary population spikes) in WT (non-tg) mice, acute ethanol increases fEPSPs and sPS and does not affect the PTP and LTP in Il-6tg mice (Hernandez et al. 2016; Bray et al. 2013). These studies on IL-6tg mice suggest possible mechanisms mediating IL-6 and ethanol interactions, particularly following the ethanol-induced increase in IL-6 levels in the brain.

6 Interleukin-10

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that negatively regulates inflammation. IL-10 is expressed in the brain; specifically, it is produced by microglia, astrocytes, and neurons (Kwilasz et al. 2015). IL-10 binds to its cognate cell surface receptor, a heterotetrameric complex consisting of two ligand-binding IL-10 receptor 1 (IL-10R1) chains and two accessory IL-10 receptor 2 (IL-10R2) chains (Kwilasz et al. 2015; Fickenscher et al. 2002) also expressed in glia and neurons. This interaction leads to the activation of downstream signaling cascades including the JAK/STAT3 and PI3-K/Akt pathways (Kwilasz et al. 2015; Fickenscher et al. 2002; Sharma et al. 2011; Norkina et al. 2007) and ultimately results in diverse biological effects in the brain such as limiting the synthesis of

pro-inflammatory mediators and reducing cytokine receptor expression and activation (Curtale et al. 2013), neuroprotection (Sharma et al. 2011; Segev-Amzaleg et al. 2013), and modulation of synaptic structure and activity (Lim et al. 2013; Suryanarayanan et al. 2016). At the cellular level, IL-10 regulates GABAergic transmission in the hippocampal (dentate gyrus) neurons via both pre- and postsynaptic mechanisms; IL-10 decreases mIPSCs and tonic GABA currents, and its postsynaptic mechanisms of actions are mediated by PI3K pathways (Suryanarayanan et al. 2016).

6.1 IL-10 and Alcohol

IL-10 is implicated in alcoholism in humans. Human genetic studies show that a -592C>A polymorphism in the IL-10 gene is associated with alcoholism (Marcos et al. 2008). Further, IL-10R2 levels are decreased in the CeA and cortex of alcoholics (Ponomarev et al. 2012). Notably, IL-10 regulates SOCS (suppressor of cytokine signaling), and SOCS mRNA levels are also decreased in the CeA and cortex of alcoholics (Ponomarev et al. 2012).

IL-10 expression and signaling are altered in several CNS pathologies (Kwilasz et al. 2015). Expression studies show that a single intoxicating dose of ethanol increases IL-10 content in rat hippocampus and primary cultured cortical neurons (Suryanarayanan et al. 2016), 24-h ethanol exposure increases IL-10 production by human monocytes (Norkina et al. 2007), 4-day binge ethanol exposure results in protracted increases in IL-10 levels in the rat hippocampus (Marshall et al. 2013), and 12-day withdrawal after chronic ethanol exposure increases IL-10 content in the rat hippocampus, prefrontal cortex, and brainstem (Schunck et al. 2015). In contrast, 4-day binge drinking in the dark paradigm decreases IL-10 levels in the mouse BLA, but not in the CeA, and IL-10 infusion into the BLA, but not the CeA, decreases binge-like drinking (Marshall et al. 2017). A 10-day binge ethanol exposure decreases mouse brain IL-10 levels (Qin et al. 2008). The differential effects on IL-10 expression are likely due to differences between species, animal models, and examination of region-specific versus whole brain changes. Despite the growing body of evidence on an important role of IL-10 in the regulation of alcohol-related behaviors, particularly binge drinking, the mechanistic and functional aspects of IL-10 and ethanol interactions are very limited.

7 Tumor Necrosis Factor-Alpha

Tumor necrosis factor-alpha (TNF-α) is a member of the TNF superfamily and is central to the innate immune response and maintenance of homeostasis at the cellular, tissue, and organism levels. In the CNS, TNF-α displays pro-inflammatory effects and is considered a major mediator of the secondary CNS damage following acute injury and during chronic inflammation. However, TNF-α also exerts essential beneficial functions in the CNS. Its potent pro-inflammatory effects require very tight temporal

and spatial control, as dysregulation of TNF-α production and activity can trigger cell death and tissue degeneration (Probert 2015). TNF-α is produced in two bioactive forms transmembrane (tmTNF) and soluble (solTNF) that differ in their biological activity and intracellular signaling (Kriegler et al. 1988). In general, solTNF has systemic inflammatory effects and is necessary for optimization of the immune response, whereas tmTNF mediates a subset of beneficial TNF-α activities and only basic immune responses (Probert 2015). The TNF-α system has two receptors – TNFR1 and TNFR2. While both TNF-α forms bind to TNFR1, tmTNF is the sole ligand for TNFR2 (Grell et al. 1995). TNFR1 is ubiquitously and constitutively expressed, and its activation induces pro-inflammatory signaling through the NF-kB and AP1 transcription factors (Walczak 2011). TNFR2 expression is restricted to immune cells, endothelial cells, and CNS cells – including neurons, astrocytes, and oligodendrocytes. Activation of TNFR2 leads mainly to induction of pro-survival signals mediated by Akt and NF-kB signaling pathways (Medvedev et al. 1994; Rao et al. 1995). Under physiological conditions, TNF-α plays an important role in the regulation of homeostatic processes such as synaptic scaling and plasticity (Stellwagen and Malenka 2006; Turrigiano 2008; Kaneko et al. 2008) and regulation of inhibitory and excitatory neurotransmission (Vezzani and Viviani 2015). Under pathological conditions in the CNS, TNF-α has both protective and pro-inflammatory effects (Probert 2015). For example, a mechanism by which TNF-α mediates neurotoxicity is by inhibiting glutamate uptake causing glutamate excitotoxicity (Zou and Crews 2005).

Similar to IL-1β and IL-6, TNF-α has neuromodulatory effects in the CNS. TNF-α enhances Na^+ channels and has mixed effects on voltage-gated Ca^{2+} channels (solTNF-α decreasing and mTNF-α increasing Ca^{2+} currents) (Vezzani and Viviani 2015). Presynaptically, TNF-α increases action potential-dependent spontaneous excitatory postsynaptic currents (sEPSCs) in corticostriatal projections, through AMPA receptors (Musumeci et al. 2011). Also, TNF-α can modify extracellular glutamate levels indirectly by inducing glutamate release from microglia (Takeuchi et al. 2006) and astrocytes (Bezzi et al. 2001) and by inhibiting glutamate uptake by astrocytes (Zou and Crews 2005). TNF-α-TNF-R1 signaling preferentially affects AMPARs in a brain region-specific manner. TNF-α facilitates AMPAR-mediated glutamatergic transmission and enhances neuronal excitability in the hippocampus, cortex, amygdala, and spinal cord (Stellwagen et al. 2005; He et al. 2012; Ferguson et al. 2008). Notably, activation of the CB_1 cannabinoid receptor reverses TNF-α effects on AMPAR (Zhao et al. 2010). In the striatum, however, TNF-α induces the internalization of GluR1-GluR2 AMPAR subunits leading to a decrease in the excitatory drive on inhibitory GABA neurons. Also, TNF-α promotes the endocytosis of GABA-A receptors (subunits b2/3) (Stellwagen et al. 2005). Thus, TNF-α effects on glutamate and GABA receptors lead to enhanced neuronal excitability and in some instances to excitotoxicity (Stellwagen and Malenka 2006; Stellwagen et al. 2005; Beattie et al. 2002; Leonoudakis et al. 2004). In the CeA, TNF-α increases the amplitude of mEPSCs via the PI3-K signaling pathway but does not affect mEPSC frequency, suggesting a predominantly postsynaptic mechanism of action. Further, TNF-α increases the firing of CeA neurons through its action on glutamate receptors

(Knapp et al. 2011; Ming et al. 2013). Finally, TNF-α increases the frequency of mIPSC, indicating an increase in presynaptic GABA release, and this effect is blocked by a CRF_1 antagonist and minocycline, which is an inhibitor of glial activation (Ming et al. 2013). These findings indicate that TNF-α interacts and modulates key neurotransmitters (GABA and glutamate) and neuropeptide (CRF) systems involved in alcohol-related behaviors (Roberto et al. 2012).

7.1 TNF-α and Alcohol

Elevated plasma levels of TNF-α in alcoholics are associated with increased craving and relapse to drinking (Kiefer et al. 2002). In contrast to IL-1β and IL-6 cytokines, genomic studies in rodents did not find alterations in TNF-α gene expression in alcohol-preferring mice (Mulligan et al. 2006). In general, TNF-α levels are predominantly decreased following acute ethanol treatment and increased after chronic ethanol exposure. Intracerebroventricular (i.c.v.) and intra-CeA administration of TNF-α before a single chronic ethanol exposure and ethanol withdrawal sensitizes ethanol withdrawal-induced anxiety-like behavior (Breese et al. 2008; Knapp et al. 2011), and this effect is mediated by CRF_1, as a CRF_1 antagonist reduces the TNF-α induced elevation of withdrawal-induced anxiety (Knapp et al. 2011). The interactions of TNF-α and the CRF system particularly in the CeA, where CRF_1 is known to mediate ethanol effects on GABAergic transmission, may represent one of the mechanisms involved in TNF-α-induced modulation of the synaptic transmission. However, the mechanisms and functional consequences of TNF-α and its interaction with ethanol on neuromodulation are not known.

8 Chemokine Ligand 2

The chemokine ligand 2 (CCL2), also known as monocyte chemotactic protein 1 (MCP-1), is a member of the monocyte chemoattractant protein (MCP) family. CCL2 is a vital chemokine that controls the migration and infiltration of monocytes/macrophages (Reaux-Le Goazigo et al. 2013). In the brain, CCL2 is produced mainly by astrocytes and microglia and to a lesser extent by endothelial cells (Semple et al. 2010). The neuronal expression of CCL2 is present in several brain regions including the cortex, hippocampus, hypothalamus, substantia nigra, and cerebellum (Banisadr et al. 2005a). Importantly, the expression of CCL2 colocalizes with classical neurotransmitters, particularly acetylcholine and dopamine (Banisadr et al. 2005a), and cell depolarization can induce Ca^{2+}-dependent CCL2 release (Jung et al. 2008; Dansereau et al. 2008). Compared to CCL2, its receptor CCR2 is expressed by resident immune cells, such as microglia (Conductier et al. 2010; Yamasaki et al. 2012). Moreover, CCR2 production is also found in cultured rat microglia (Boddeke et al. 1999), human fetal astrocytes (Andjelkovic et al. 2002), and neurons of the adult rat brain (Rostene et al. 2007), mainly from the cortex, hippocampus, hypothalamus, amygdala, substantia nigra, ventral tegmental area

(VTA), and cerebellum (Banisadr et al. 2005b). There are two splice variants of CCR2 – CCR2A and CCR2B. The splice variants are expressed in different immune cells, and they activate different signaling pathways and exert distinct actions. CCR2, as a Gαi class G-protein-coupled receptor (Kuang et al. 1996), signals through inhibition of adenylate cyclase and PI3-K, MAPK, and protein kinase C pathways (Wain et al. 2002; Old and Malcangio 2012; Dawson et al. 2003). CCL2 enhances neuronal excitability and excitatory synaptic transmission in CA1 hippocampal neurons via presynaptic mechanisms (Zhou et al. 2011). Importantly, CCR2 has both pro-inflammatory and anti-inflammatory actions (Reaux-Le Goazigo et al. 2013), and CCL2-CCR2 are involved in some physiological processes and the pathogenesis of neurodegenerative disorders and AUD.

8.1 CCL2 and Alcohol

CCL2 levels are elevated in several brain regions (e.g., hippocampus and cortex) in postmortem tissue of human alcoholics (Lewohl et al. 2000; He and Crews 2008) and the cerebrospinal fluid (CF) of alcohol-dependent human subjects (Umhau et al. 2014). Similarly, ethanol exposure and withdrawal increases levels of CCL2 in several brain regions (see Table 1) (Knapp et al. 2011, 2016; Qin et al. 2008; Freeman et al. 2012; Kane et al. 2013, 2014; Vetreno et al. 2013; Chang et al. 2015; Drew et al. 2015; Pascual et al. 2015). Indeed, alterations in the CCL2 system affect ethanol intake and motivation as mice deficient in Ccl2 or Cclr2, the genes encoding CCL2 and its receptor CCLR2, drink less ethanol and show reduced ethanol-induced aversion (Blednov et al. 2005). There is no significant difference in ethanol intake between ethanol nondependent CCL2-tg (mice overexpressing CCL2 in astrocytes) and their control WT (non-tg) mice, whereas ethanol-dependent CCL2-tg mice drink less than the dependent non-tg mice (Gruol 2016). Notably, chronic infusions of CCL2 result in long-lasting heightened ethanol intake in rats suggesting that persistent exposure to CCL2 may be required for CCL2/alcohol interactions (Valenta and Gonzales 2016). Interestingly, CCL2-tg mice did not show acute alcohol-induced impairments in contextual learning that are observed in non-tg mice (Bray et al. 2013). However, ethanol induced a spatial learning impairment in nondependent CCL2-tg mice but not in nondependent non-tg mice. Overexpression of CCL2 has a protective effect against alcohol-induced impairments in associative learning (Gruol 2016). Like IL-1β and TNF-a, intracerebral injection of CCL2 before ethanol exposure and withdrawal elevates ethanol withdrawal-induced anxiety-like behavior (Breese et al. 2008). At the cellular level, CCL2-tg mice are resistant to the depressing effects of acute alcohol (20–60 mM) on hippocampal LTP in non-tg mice. CCL2 can enhance neuronal excitability and excitatory synaptic transmission in CA1 hippocampal neurons via presynaptic mechanisms (Bray et al. 2013). These studies on transgenic animals targeting CCL2 have significantly advanced our understanding of the potential role of CCL2 in the neuropathology of AUD.

Mechanistically, CCL2/CCR2 system involvement in the neurobiology of AUD includes interactions with other neurotransmitter and neuropeptide systems, particularly CRF and the orexigenic peptide MCH (melanin-concentrating hormone). CCL2 is expressed in cholinergic and dopaminergic neurons (Banisadr et al. 2005a), and it modulates neuronal activity and synaptic transmission (Guyon et al. 2009; Apartis et al. 2010). CCL2 levels in the CeA and VTA are increased in alcohol-preferring P rats compared to non-preferring rats, and CCL2 in these brain regions, but not in the ventral pallidum, mediate binge drinking in P rats. Importantly, CRF mediates feedback regulation of TLR4 (Toll-like receptor 4) and CCL2 signaling in the CeA and VTA during ethanol consumption (June et al. 2015) suggesting that CRF, TLR4, and CCL2 in these regions regulate the initiation of excessive drinking (June et al. 2015). Moreover, prenatal exposure to ethanol increases later adolescent ethanol drinking which is associated with increased CCR2 levels and increased density of neurons co-expressing CCR2 and MCH in the lateral hypothalamus (Chang et al. 2015). As both CCR2 and MCH are believed to promote ethanol intake, these findings suggest that these systems may work together to promote ethanol drinking. Although our understanding of the mechanisms mediating CCL2's contribution to AUDs has advanced, there are still many unknowns regarding CCL2's regulation of synaptic transmission in other alcohol-related brain regions.

9 Conclusion

The role of the neuroimmune system and cytokines in the neurobiology of AUDs is supported by several lines of evidence. Ethanol-induced cytokine responses in the CNS are dynamic and depend on multiple factors including the duration and amount of ethanol exposure, sex, brain region, cellular specificity, and history of previous immune challenges (e.g., infection, trauma, stress, etc.). Cytokines contribute to the neuroadaptive changes in the CNS induced by ethanol exposure through their direct and indirect effects on all CNS cell types, which lead to the modulation of neuronal activity, glia cells, neurogenesis, and potentially neurodegeneration.

Although our understanding of the role of key cytokines in the ethanol-induced immune response has advanced, there are still many unanswered questions especially regarding the therapeutic implications of targeting cytokines and their downstream signaling pathways. The critical role of the neuroimmune system in the neuropathology of AUD suggests its potential to be targeted for the development of new treatments for AUDs. Currently, the focus of preclinical research is on inhibiting the alcohol-induced neuroimmune response and associated alcohol-related behaviors, particularly alcohol drinking. The strategies involve targeting individual components of the neuroimmune system (e.g., TLR4 (Wu et al. 2012), IL-1R1 (Wu et al. 2011), or IL-10 (Marshall et al. 2017)) or to use drugs that simultaneously target several inflammatory pathways as well as other brain signaling systems (e.g., peroxisome proliferator-activated receptor agonists (fenofibrate, pioglitazone, tesaglitazar, bezafibrate) (Stopponi et al. 2011, 2013; Blednov et al. 2015b),

phosphodiesterase inhibitors (e.g., ibudilast, rolipram) (Bell et al. 2015; Blednov et al. 2014), and naloxone/naltrexone (Kamdar et al. 2007; Tomie et al. 2013)). Regarding cytokines, preclinical studies suggest that activation or increased expression of anti-inflammatory cytokines such as IL-1Ra and IL-10 might have therapeutic value. There are, however, several challenges in targeting the neuroimmune system for the development of therapeutic strategies for alcoholism: (1) different inflammatory pathways seem to be critical for different stages of alcohol addiction and alcohol-related behaviors (Robinson et al. 2014), (2) the peripheral immune system is compromised in human alcoholics (Szabo and Saha 2015), and (3) the neuroimmune response has both neurotoxic and neuroprotective roles. And thus, strategies based solely on blocking the neuroimmune system may be counterproductive (Du et al. 2017). Understanding the role and mechanisms of action of individual components of the neuroimmune systems in the development and maintenance of alcohol addiction and relapse will be crucial for the identification of new, more target-specific and efficacious therapies for AUD.

Acknowledgments This publication was supported by National Institutes of Health grants U01 AA013498, AA015566, AA006420, AA017447, T32 AA007456, and R01 AA021491 from the National Institute on Alcohol Abuse and Alcoholism and the Pearson Center for Alcoholism and Addiction Research. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Part IV

Neuropeptides and Genomics

Corticotropin-Releasing Factor (CRF) Neurocircuitry and Neuropharmacology in Alcohol Drinking

Allyson L. Schreiber and Nicholas W. Gilpin

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A. L. Schreiber
Department of Physiology, Louisiana State University Health Science Center New Orleans, New Orleans, LA, USA

N. W. Gilpin (✉)
Department of Physiology, Louisiana State University Health Science Center New Orleans, New Orleans, LA, USA

Neuroscience Center of Excellence, Louisiana State University Health Science Center New Orleans, New Orleans, LA, USA
e-mail: ngilpi@lsuhsc.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_86

Abstract

Alcohol use is pervasive in the United States. In the transition from nonhazardous drinking to hazardous drinking and alcohol use disorder, neuroadaptations occur within brain reward and brain stress systems. One brain signaling system that has received much attention in animal models of excessive alcohol drinking and alcohol dependence is corticotropin-releasing factor (CRF). The CRF system is composed of CRF, the urocortins, CRF-binding protein, and two receptors – CRF type 1 and CRF type 2. This review summarizes how acute, binge, and chronic alcohol dysregulates CRF signaling in hypothalamic and extra-hypothalamic brain regions and how this dysregulation may contribute to changes in alcohol reinforcement, excessive alcohol consumption, symptoms of negative affect during withdrawal, and alcohol relapse. In addition, it summarizes clinical work examining CRF type 1 receptor antagonists in humans and discusses why the brain CRF system is still relevant in alcohol research.

Keywords

Alcohol dependence · Alcohol use disorder · Binge drinking · Corticotropin-releasing factor · Urocortin

1 Problematic Alcohol Use in Humans

Alcohol use is pervasive in the United States, with ~88% of adults 18 years or older reporting alcohol use at some time during their life and ~55% of adults reporting alcohol use within the past month (CBHSQ 2016). With the high prevalence of alcohol drinking, it is unsurprising that alcohol accounts for ~4% of global disease burden and is the fourth leading preventable cause of death in the United States (Mokdad et al. 2004). Therefore, there is an urgent need to understand the neurobiological processes that underlie the transition from moderate controlled alcohol use to problematic alcohol use in humans.

In the transition from nonhazardous drinking to hazardous drinking and alcohol use disorder (AUD), neuroadaptations occur within brain reward and brain stress systems. Initial alcohol use is driven by positive reinforcement, that is, drinking for the euphoric or rewarding effects of alcohol, and brain reward pathways are predominantly activated in this stage of alcohol use (Koob 2003). Intermittent bouts of binge alcohol consumption occur during the transition from moderate use to alcohol dependence (Koob and Le Moal 1997). During this time, individuals transition from drinking alcohol for its positive reinforcing effects to drinking alcohol for its negative reinforcing effects, in many cases to relieve the negative affective symptoms that define alcohol withdrawal (Koob 2003; Koob and Le Moal 1997). One brain signaling system that has received much attention in animal models of excessive alcohol drinking and alcohol dependence is corticotropin-releasing factor (CRF), a pro-stress neuropeptide that is dysregulated by chronic high-dose alcohol exposure and that appears to contribute to binge alcohol drinking, alcohol dependence, and alcohol relapse.

2 Introduction to Brain CRF System

2.1 CRF and Urocortins

2.1.1 Corticotropin-Releasing Factor (CRF)

CRF is a 41-amino acid neuropeptide that is evolutionarily conserved across species (Vale et al. 1981). Within the central nervous system, CRF acts as a neuromodulator at both pre- and postsynaptic sites (Lowry and Moore 2006). In general, neuromodulators (like CRF) work at G-protein-coupled receptors, and they have longer-lasting effects than classical neurotransmitters (van den Pol 2012). Such neuromodulators may enhance or attenuate neuronal activity by modulating the activity of ion channels or by increasing or decreasing the activity of classical neurotransmitters via direct actions on peptide receptors (van den Pol 2012). CRF is widely expressed in the brain, including in the cortex, hypothalamus, thalamus, hippocampus, midbrain, and locus coeruleus (LC) (Merchenthaler 1984; Peng et al. 2017). The highest density of CRF neurons is in the paraventricular nucleus (PVN) of the hypothalamus and the extended amygdala, particularly the central amygdala (CeA) and bed nucleus of the stria terminalis (BNST; Dunn and Berridge 1990; Merchenthaler 1984; Peng et al. 2017). Importantly, CRF neurons are heterogeneous, co-expressing different molecules, having different electrophysiological properties, and differing in soma shape depending on the region (Dabrowska et al. 2013a, b; Peng et al. 2017).

Hypothalamic CRF projections modulate endocrine and autonomic responses to stress. CRF released from glutamatergic parvocellular neurons in the PVN is the primary activator of the hypothalamic-pituitary-adrenal (HPA) axis (Rivier and Vale 1983; Vale et al. 1981). Acting as a hormone, CRF is released from the PVN into the median eminence, where it travels to the pituitary, and binds receptors on corticotrophs, thereby increasing the synthesis and release of adrenocorticotropic hormone (ACTH). ACTH travels in systemic circulation and increases glucocorticoid synthesis and release from the adrenal gland, thereby initiating the endocrine stress response. After initiation of the endocrine stress response, glucocorticoids feed back onto CRF cells in the PVN and other brain regions (e.g., hippocampus and cortex) to decrease CRF production. During stress, hypothalamic CRF neurons can synthesize and co-release arginine vasopressin (AVP), which can increase ACTH release from corticotrophs in the anterior pituitary (Sawchenko et al. 1984). In addition, a majority of CRF neurons in the PVN express transcripts for oxytocin, suggesting that these neurons have multiple effects according to physiological demand (Dabrowska et al. 2013a).

Extended amygdala CRF pools participate in coordination of visceral, behavioral, and emotional responses to stress. CRF is particularly abundant in the lateral division of the CeA and the dorsolateral division of the BNST (Fig. 1; Pomrenze et al. 2015; Shimada et al. 1989). CeA and oval BNST (oBNST) CRF neurons share many similarities with each other and appear to be different from CRF neurons in the PVN. For example, while glucocorticoids negatively regulate CRF transcription in the PVN, glucocorticoids increase CRF production in a positive feedback loop in the

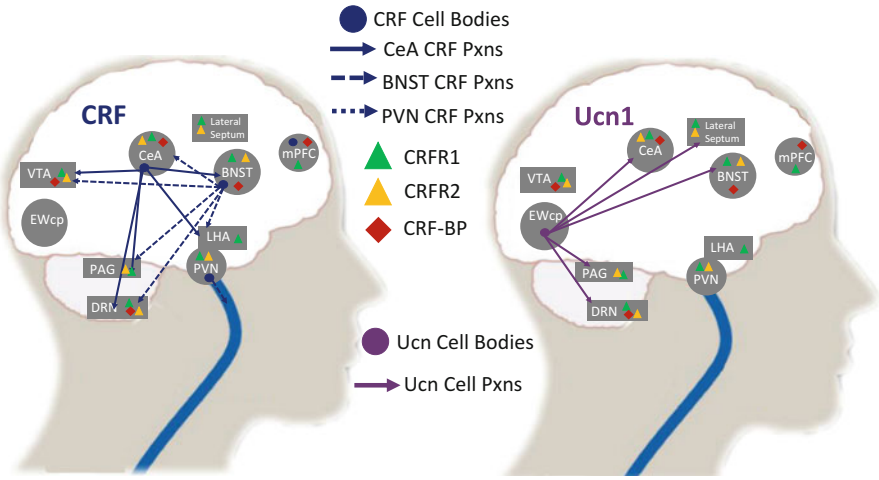

Fig. 1 Distribution and projection of CRF, Ucn1, and CRFRs in alcohol-related regions. Corticotropin-releasing factor (CRF) is more widely expressed than urocortin 1 (Ucn1); CRF type 1 receptors (CRFR1, green triangle) are expressed widely throughout the brain, while CRF type 2 receptors (CRFR2, yellow triangle) have a more restricted distribution. Corticotropin-releasing factor-binding protein (CRF-BP, red diamond) is expressed in most brain regions that express CRF, Ucn1, and CRFRs. *CeA* central amygdala, *DRN* dorsal raphe nucleus, *EWcp* centrally projecting Edinger-Westphal nucleus, *LHA* lateral hypothalamus, *mPFC* medial prefrontal cortex, *PAG* periaqueductal gray, *PVN* paraventricular nucleus of the hypothalamus

CeA and BNST (Shepard et al. 2000). CRF is typically expressed in GABAergic interneurons with medium spiny neuron morphology in both the CeA and the BNST (Phelix and Paull 1990). Interestingly, CRF neurons in the CeA project to BNST, CRF neurons in the BNST project to CeA, and these two CRF neuron pools project to many of the same downstream regions including the lateral hypothalamus, ventrolateral periaqueductal gray (PAG), dorsal raphe nucleus (DRN), and ventral tegmental area (VTA) (Dabrowska et al. 2016; Pomrenze et al. 2015). CRF neurons in CeA and BNST also co-express overlapping molecules, including but not limited to dynorphin, neurotensin, and somatostatin (Pomrenze et al. 2015; Shimada et al. 1989). In addition, CRF neurons in CeA and oBNST both co-express striatal-enriched protein tyrosine phosphatase (STEP; Dabrowska et al. 2013b). STEP may function as an indirect marker of neuronal activation in the CeA and BNST because it dephosphorylates neuronal activation markers (e.g., pERK), and it is expressed in 98% of CRF neurons in the oBNST and 94% of CRF neurons in the CeA (Dabrowska et al. 2013b).

2.1.2 Urocortins (Ucns)

The urocortins (Ucns) are more recently discovered components of the CRF system. Urocortins 1, 2, and 3 (Ucn1, Ucn2, and Ucn3) share a highly conserved structural homology, but each molecule has a unique distribution and function in the mammalian brain. Although the exact physiological function of the Ucn system remains

unclear, it appears to be involved in modulation of physiological processes that include stress response, osmoregulation, energy expenditure, food intake, and immune function (Fekete and Zorrilla 2007).

Ucn1 is expressed primarily in non-cholinergic cells in the centrally projecting Edinger-Westphal nucleus (EWcp), a subdivision of the Edinger-Westphal nucleus that is not involved in autonomic responses (Bittencourt et al. 1999; Ryabinin et al. 2012). Ucn1 fibers project to lateral septum, DRN, supraoptic nucleus, PVN, PAG, Edinger-Westphal nucleus, BNST, CeA, and medial amygdala (MeA; Fig. 1; Fekete and Zorrilla 2007; Pan and Kastin 2008; Ryabinin et al. 2012). In response to acute stress, Ucn1 shows rapid induction that is mediated by glucocorticoids (Koob and Heinrichs 1999; Weninger et al. 2000). Because Ucn1 binds to both CRF receptor subtypes (discussed below), its physiological function is not completely understood. It has been hypothesized that midbrain Ucn1 neurons play a role in sympathetic-mediated behavioral responses to stress, including increases in anxiety-like behavior and decreases in food consumption (Koob and Heinrichs 1999; Pan and Kastin 2008). Others have postulated that Ucn1 expression may be important for balancing activation of CRF receptor subtypes during stress (Ryabinin et al. 2012). In this hypothesis, CRF and Ucn1 signaling at CRFR1 initiate the sympathetic, endocrine, and behavioral responses to stress, and Ucn1 signaling at CRFR2 may also mediate the later adaptive phases of stress (Ryabinin et al. 2012).

Ucn2 (also known as stresscopin-related peptide) is expressed in the PVN; supraoptic nucleus; LC; trigeminal, facial, and hypoglossal motor nuclei; and meninges (Dunn and Berridge 1990; Reyes et al. 2001; Ryabinin et al. 2012). Projection targets of Ucn2 fibers are not known (Fekete and Zorrilla 2007), but it is hypothesized that Ucn2 projections from LC to DRN increase depressive-like behavior by modulating serotonergic signaling (Fekete and Zorrilla 2007). In addition, Ucn2 may modulate basal HPA circadian amplitude in females by modulating AVP levels (Chen et al. 2006).

Ucn3 (i.e., stresscopin) is the most widely expressed urocortin. It is expressed in the medial preoptic area, perifornical area, BNST, MeA, ventral premammillary nucleus, superior olivary nucleus, and parabrachial nucleus (Ryabinin et al. 2012). Projection targets for Ucn3 cells include the lateral septum and ventromedial hypothalamus, both of which contain high levels of CRF receptor type 2 (Hillhouse and Grammatopoulos 2006). Ucn3 modulates food intake and basal neuroendocrine regulation (Hillhouse and Grammatopoulos 2006).

2.2 CRF Receptors

There are two CRF receptor subtypes in the mammalian central nervous system – CRF type 1 receptor (CRFR1) and CRF type 2 receptor (CRFR2). Both CRFR1 and CRFR2 are G_s-protein-coupled receptors, and binding of endogenous ligands to these receptors activates adenylate cyclase, increases cAMP, and increases protein kinase A (PKA) signaling (Chen and Du 1996). In addition, these receptors are

bound to various structural proteins that modulate CRF signaling according to brain region and physiological state (Henckens et al. 2016).

2.2.1 CRFR1

CRFR1s are widely expressed, with high concentrations in the anterior hypophysis, cerebral cortex, cerebellum, BNST, CeA, MeA, BLA, hippocampus, globus pallidus, and VTA (Fig. 1; Chalmers et al. 1995; Henckens et al. 2016; Van Pett et al. 2000). CRFR1 binds both CRF and Ucn1 with high affinity (Bittencourt et al. 1999), and its expression corresponds to areas where there is high expression of CRF and Ucn1 cell bodies and projection fibers. Canonically, CRFR1 has been considered to be "pro-stress," because increases in CRFR1 signaling are anxiogenic (Dunn and Berridge 1990), whereas antagonizing or knocking out CRFR1 reduces anxiety-like behavior (Henckens et al. 2016; Muller et al. 2003; Timpl et al. 1998; Zorrilla et al. 2002).

2.2.2 CRFR2

CRFR2s exhibit expression that is restricted to subcortical brain regions including the amygdala, BNST, lateral septum, and DRN (Fig. 1; Chalmers et al. 1995; Van Pett et al. 2000). CRF has a much lower affinity for CRFR2 than for CRFR1; however, the Ucns all show high affinity for CRFR2, and they appear to be the primary endogenous ligand for this receptor. As such, regions that show high CRFR2 expression also show high Ucn expression and/or receive projections from Ucn-rich brain areas. Two hypotheses exist to explain the role of CRFR2 activation in anxiety-like behavior (Henckens et al. 2016): the first is that CRFR2 activation counteracts the initial stress response and maintains homeostasis (Henckens et al. 2016; Hillhouse and Grammatopoulos 2006); the second is that CRFR1 and CRFR2 mediate different aspects of the stress response, with CRFR1 mediating active defensive behavior and CRFR2 mediating passive coping behavior and depression-like responses (Henckens et al. 2016).

2.3 CRF-Binding Protein

CRF-binding protein (CRF-BP) is a 37kd secreted glycoprotein that binds CRF and Ucn1. In humans, CRF-BP binds 40–90% of CRF, and its expression is tenfold higher than CRF levels in most regions of the human brain (Hillhouse and Grammatopoulos 2006; Suda et al. 1988). CRF-BP expression and synthesis is regulated by stress, CRF, and glucocorticoids (Westphal and Seasholtz 2006). In addition, CRF-BP is often co-localized with CRF or CRFRs in the brain, with particularly high concentrations in CeA and BNST (Fig. 1; Chan et al. 2000; Potter et al. 1992; Westphal and Seasholtz 2006).

Once CRF-BP binds CRF, it can inhibit or facilitate CRF activity. For example, CRF-BP can reduce CRFR activation by sequestering CRF or Ucn1 and/or targeting them for degradation (Ketchesin and Seasholtz 2015). Conversely, CRF-BP can increase CRF signaling by binding CRF and interacting with CRFRs, as recently

shown for CRFR2 in the VTA (Albrechet-Souza et al. 2015; Ungless et al. 2003). In addition to modulating the activity of CRF and its receptors, CRF-BP may affect neuronal activity independent of CRF and CRFRs. For example, intraventricular administration of CRF-BP increases neuronal activation in CRFR-expressing cells but also in CRF-BP-expressing cells that do not co-express CRF or CRFR (Chan et al. 2000).

3 Alcohol Effects on CRF Signaling

3.1 Acute Alcohol Effects on the CRF System

3.1.1 CRF

Acute alcohol activates the HPA axis by inducing CRF cell activation in the hypothalamus (Rivier and Lee 1996). Acute alcohol increases CRF heteronuclear RNA (hnRNA) in the PVN, suggesting increased CRF synthesis (Rivier and Lee 1996); however, there is not a clear increase in CRF mRNA following acute alcohol in vivo (Rivier and Lee 1996). The difference between hnRNA and mRNA could be due to presence of a large stable pool of CRF mRNA in parvocellular neurons of the PVN, which may make it hard to detect small changes, or due to unknown alcohol effects on events between gene transcription and detection of mRNA (Rivier and Lee 1996). Work done in hypothalamic cell culture has demonstrated acute alcohol-induced increases in CRF mRNA, CRF promoter activity, and CRF secretion via increases in cAMP and PKA (Li et al. 2005). In addition, in an in vitro hypothalamic preparation, acute alcohol exposure increased CRF release from neurons (Redei et al. 1988). See Table 1 for a summary of the effects of different alcohol exposure regimens on brain CRFR system signaling.

Within the CeA, high doses of acute alcohol increase CRF release 120–180 min later (Lam and Gianoulakis 2011). Interestingly, acute alcohol-induced increases in frequency of spontaneous mini inhibitory postsynaptic currents (IPSCs) in the CeA are mediated by CRFR1 signaling through both protein kinase Cε and PKA pathways (Bajo et al. 2008; Cruz et al. 2012; Roberto et al. 2010). Therefore, acute alcohol increases CRF release in CeA, which in turn increases CeA inhibitory transmission, suggesting that CRF modulates acute alcohol effects on synaptic transmission in CeA.

3.1.2 Ucn1

The Ucn1 system is activated by acute alcohol and may mediate acute alcohol effects on other brain signaling systems. Acute alcohol increases activation of Ucn1 cells in the EWcp, as measured by c-fos (Ryabinin et al. 1995, 1997, 2003). This acute alcohol-induced activation of Ucn1 neurons in the EWcp is slow to habituate to repeated bouts of alcohol exposure, as evidenced by increased c-fos expression in Ucn1 neurons in the EWcp following repeated alcohol self-administration (Ryabinin et al. 2003; Turek and Ryabinin 2005). The observed lack of tolerance to acute alcohol effects suggests that Ucn1 neurons may be involved in behaviors that

Table 1 Alcohol effects on CRF signaling

	PVN	CeA	BNST	VTA	mPFC	EWcp	Lateral septum	DRN
Low-level/acute alcohol exposure effect on								
CRF	↑ hnRNA, activity	↑ Release	?	?	↔ Cell number	?	?	?
Binge-like alcohol consumption effect on								
CRF	*Adolescents* ?	*Adolescents* ↓ (Operant binge) cell number	*Adolescents* ↔ (Operant binge) cell number	*Adolescents* ?	*Adolescents* ?	*Adolescents* ?	*Adolescents* ?	*Adolescents* ?
	Adults ↔ (DID) mRNA ↓ (Escalation model) binding and signaling	*Adults* ↑ (DID) CRF-ir ↔ (DID) mRNA	*Adults* ↔ (DID) mRNA	*Adults* ↑ (DID) mRNA	*Adults* ?	*Adults* ?	*Adults* ↔ (DID) mRNA	*Adults* ?
CRF-BP	?	↔ (DID) mRNA	↔ (DID) mRNA	↓ (DID) mRNA	↓ (DID) mRNA	↑ (Escalation model) mRNA	?	?
Chronic alcohol/dependence effect on								
CRF	*Intoxication* ↑ mRNA	*Intoxication* ↑ mRNA	*Intoxication* ↔ release	*Intoxication* ?	*Intoxication* ?	*Intoxication* ?	*Intoxication* ?	*Intoxication* ?
	Acute WD ↓ Content, mRNA	*Acute WD* ↑ Release ↓ Content	*Acute WD* ↑ Release ↓ Content	*Acute WD* ?	*Acute WD* ↑ Cell number	*Acute WD* ?	*Acute WD* ?	*Acute WD* ?
	Protracted WD ?	*Protracted WD* ↑ mRNA	*Protracted WD* ?	*Protracted WD* ?	*Protracted WD* ?	*Protracted WD* ?	*Protracted WD* ?	*Protracted WD* ?
CRFR1	↑ mRNA (blunted compared to acute)	↑ mRNA (mice) ↔ mRNA (rats)	↔ mRNA (rats)	?	?	?	?	?
CRFR2	?	↔ mRNA	↔ mRNA	?	?	?	↑ mRNA	↑ mRNA

↑ increase in expression or signaling due to alcohol, ↔ no change in expression or signaling due to alcohol, ↓ decrease in expression or signaling due to alcohol, ? unknown

CRF corticotropin-releasing factor, *CRF-BP* CRF-binding protein, *CRFR1* CRF receptor type 1, *CRFR2* CRF receptor type 2, *PVN* paraventricular nucleus of the hypothalamus, *CeA* central amygdala, *BNST* bed nucleus of stria terminalis, *VTA* ventral tegmental area, *mPFC* medial prefrontal cortex, *EWcp* centrally projecting Edinger-Westphal nucleus, *DRN* dorsal raphe nucleus

accompany prolonged alcohol exposure (Weitemier and Ryabinin 2005). See Table 3 for a summary of the effects of different alcohol exposure regimens on brain Ucn system signaling.

Overall, acute alcohol exposure leads to activation of hypothalamic and extra-hypothalamic CRF systems and activation of Ucn1 cells within the EWcp. More work is needed to determine if acute alcohol effects on brain CRF signaling change after repeated low-level exposures and if/how they contribute to escalation of alcohol use.

3.2 Binge Alcohol Effects on the CRF System

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as a pattern of ethanol consumption that leads to blood alcohol concentrations (BACs) of 80 mg/dL or above (NIH-NIAAA 2004), which is usually about four drinks in about 2 h for women and five or more drinks for men. Binge alcohol consumption is associated with increased risk to develop AUD and is observed in populations that do and do not meet criteria for an AUD diagnosis (Deas and Brown 2006; Lai et al. 2012). Binge alcohol consumption affects brain CRF signaling acutely post-binge, and it is hypothesized that binge alcohol drinking may induce plasticity in brain CRF systems that becomes more robust and more rigid with repeated binge-like drinking episodes (Lowery-Gionta et al. 2012).

3.2.1 CRF

Different models of binge drinking have produced conflicting results regarding binge alcohol effects on hypothalamic CRF and HPA activity. Binge-like alcohol consumption in the drinking in the dark (DID) mouse model does not change HPA axis activity following one DID session (Lowery et al. 2010). Furthermore, repeated DID cycles do not alter CRF immunoreactivity in the PVN, although corticosterone levels were not measured in this study (Lowery-Gionta et al. 2012). In contrast, in a model of intermittent alcohol homecage drinking in which *rats* achieve high levels of alcohol consumption punctuated by intermittent periods of abstinence, alcohol consumption decreases CRF binding and downstream signaling in the hypothalamus (Simms et al. 2014). This effect was attributed to the binge-like pattern of alcohol consumption in intermittent drinkers, rather than the overall amount of alcohol consumed, as animals with continuous access to alcohol had higher lifetime alcohol consumption but did not display the same changes in hypothalamic CRF binding and downstream G-protein-coupled signaling (Nielsen et al. 2012; Simms et al. 2014). The difference between the DID model and escalation model may have to do with the pattern of EtOH consumption as well as the different species used for each model. Future work will determine how repeated binge-like sessions of alcohol drinking alter hypothalamic CRF signaling and HPA activity and how these changes potentially contribute to excessive alcohol drinking and/or impact the transition to alcohol dependence.

The effects of binge-like alcohol drinking on the extra-hypothalamic CRF system depend on the age at time of alcohol exposure. Adolescence is a time of particularly high vulnerability to alcohol effects on the brain, and heavy onset early drinking is one of the strongest predictors of lifetime AUD (Chou and Pickering 1992). Binge drinking is highly prevalent in adolescents, and binge alcohol effects on adolescent brain CRF systems may affect subsequent alcohol-related behaviors (Gilpin et al. 2012). Following 14 days of binge-like alcohol consumption, adolescent male and female rats exhibit reductions in CRF cell number in the CeA, with no changes in CRF cell number in the BNST (Karanikas et al. 2013). Interestingly, adult male rats with a history of voluntary binge drinking in adolescence also exhibit reductions in CRF immunoreactivity in the CeA (Gilpin et al. 2012). This suggests that adolescent binge alcohol effects on the CeA CRF system last into adulthood (Gilpin et al. 2012) and may contribute to increased AUD vulnerability in adolescent binge drinkers (Chou and Pickering 1992).

Although binge alcohol drinking is common in adolescents, it is not a pattern of drinking seen only in adolescents. In fact, a large proportion of adults engage in binge drinking behavior (CBHSQ 2016), and binge drinking is associated with negative consequences and increased risk for AUD in adults as well as adolescents (Jennison 2004; CBHSQ 2016). In adult mice, after one and six cycles of DID, CRF-ir is increased in the CeA of mice with a history of binge-like alcohol drinking when compared to sucrose controls, suggesting that contrary to prior dogma, the CeA CRF system is recruited during early binge-like drinking episodes in animals without a chronic alcohol history (Lowery-Gionta et al. 2012). This increase in CeA CRF-ir persists 18–24 h post-binge, well after alcohol has been cleared from the blood and brain (Lowery-Gionta et al. 2012). This increase in CRF immunoreactivity may not be due to higher local CRF synthesis, as CRF mRNA was unchanged in the CeA 24 h after the last of three DID cycles (Ketchesin et al. 2016). Additionally, repeated bouts of binge-like alcohol drinking result in a reduction in the ability of CRF to enhance GABAergic transmission in the CeA (Lowery-Gionta et al. 2012), which differs from alcohol dependence effects on CRF modulation of CeA GABAergic transmission (see below). This suggests a unique functional neuroadaptation in the CeA following repeated binge cycles that may contribute to escalated alcohol use and maintenance of excessive binge-like alcohol intake. Similar to adolescents, repeated cycles of DID do not alter CRF-ir or CRF mRNA outside the CeA (i.e., in the BNST, BLA, MeA, NAc core and shell, lateral hypothalamus, or lateral septum; Ketchesin et al. 2016; Lowery-Gionta et al. 2012). Interestingly, the VTA exhibits transient increases in CRF and decreases in CRF-BP levels following acute binge cycles that normalize after repeated binge exposures (Ketchesin et al. 2016; Lowery-Gionta et al. 2012; Rinker et al. 2017). More specifically, CRF mRNA and CRF-ir in VTA are increased following one cycle of DID, but CRF-ir normalizes after six cycles of DID (CRF mRNA was not measured after six cycles; Lowery-Gionta et al. 2012; Rinker et al. 2017). CRF-BP mRNA is decreased following three cycles of DID but, like CRF-ir, returns to normal after six cycles of DID (Ketchesin et al. 2016; Lowery-Gionta et al. 2012). Overall, this suggests that binge alcohol transiently increases CRF availability

and/or activity in the VTA, which may represent a point of interaction between brain stress and reward systems and which may contribute to the transition to alcohol dependence.

3.2.2 Ucn1

The effect of binge drinking on the Ucn1 system has not been extensively studied, although recent work suggests that Ucn1 neurons within the EWcp play a role in the maintenance of high levels of alcohol consumption (Giardino et al. 2017). Following long-term intermittent alcohol drinking in which mice escalate alcohol intake to binge-like levels, mice exhibit increased mRNA levels of Ucn1 and CRF-BP in the EWcp (Giardino et al. 2017). Furthermore, in that study, alcohol intake levels were positively correlated with *fos* mRNA levels in EWcp (Giardino et al. 2017). Overall, these results suggest that binge alcohol increases activity of the brain Ucn1 system.

3.2.3 CRF-BP

Similar to Ucn1, it is largely unknown how binge-like alcohol consumption alters CRF-BP levels and its function in different brain regions. One study did not find binge alcohol effects on CRF-BP mRNA in the extended amygdala, but did find decreased CRF-BP transcript in extra-hypothalamic regions including the VTA (as mentioned earlier) and the mPFC (Ketchesin et al. 2016). Three cycles of DID binge-like alcohol drinking reduce CRF-BP mRNA in both the prelimbic (PrL) and infralimbic (IL) subdivisions of the mPFC, but this decrease is more persistent in the PrL than in the IL (Ketchesin et al. 2016). The same procedure did not alter CRF mRNA in the brain regions tested. Further work is needed to determine whether CRF-BP in mPFC plays a causal role in alcohol-related behavioral dysregulation.

Overall, binge-like alcohol consumption alters CRF signaling differently depending on the model, the brain region, and the age of exposure, with the CeA exhibiting the largest, most lasting effects. Higher CRF (and possibly Ucn1) signaling after binge-like alcohol consumption in brain reward and stress regions may contribute to increased vulnerability to addiction. Much remains unknown regarding the relationship between binge-like alcohol consumption and brain CRF signaling, including potential differences between adolescents and adults, potential lasting effects of binge alcohol drinking on brain CRF signaling, and how this latter effect may contribute to the transition to alcohol dependence.

3.3 Chronic Alcohol (i.e., Dependence) Effects on the CRF System

3.3.1 CRF

Extensive work has detailed the effects of chronic alcohol on HPA axis activity in humans with AUD (Adinoff et al. 1998; Stephens and Wand 2012). In general, humans with AUD display elevated basal ACTH and cortisol and hyporeactive HPA response to acute alcohol (as reviewed in Blaine et al. 2016). During early abstinence from alcohol, humans with AUD have low cortisol production and a blunted cortisol response to stress (Stephens and Wand 2012). Animals exposed to chronic alcohol

liquid diet exhibit alterations in HPA axis function similar to what is seen in humans, with basally increased corticosterone levels during alcohol dependence, and lower HPA activity after alcohol is removed, which persists 3 weeks into alcohol withdrawal (Rasmussen et al. 2000). Exposure to alcohol for 3–7 days leads to increased CRF gene expression and biosynthesis immediately after the alcohol exposure (Rivier et al. 1990). During acute withdrawal, chronic alcohol exposure also decreases CRF mRNA content in hypothalamic neurons without changing CRF release characteristics and decreases anterior pituitary corticotroph responses to CRF by decreasing CRF binding and adenylate cyclase in the pituitary of chronic alcohol-exposed rats (Richardson et al. 2008; Redei et al. 1988). Downregulation of CRF in the hypothalamus may be explained by negative feedback from higher circulating cortisol levels, whereas high levels of circulating cortisol increase CRF levels in the extended amygdala and may "sensitize" the extended amygdala to the effects of chronic alcohol (Koob 2010; Shepard et al. 2000).

Many studies have examined chronic alcohol effects on CRF in the CeA. Immediately following chronic ingestion of alcohol liquid diet, CRF mRNA in the CeA is increased (Lack et al. 2005). Various studies have examined CeA CRF during alcohol withdrawal and collectively report that alcohol withdrawal leads to decreased CRF immunoreactivity and increased CRF release, as measured by microdialysis, in CeA (Funk et al. 2006; Merlo Pich et al. 1995; Zorrilla et al. 2001). Specifically, CRF release peaks ~10–12 h into withdrawal in alcohol-dependent rats (Merlo Pich et al. 1995). This increase in CRF release corresponds to a decrease in CRF-ir seen during the first day of withdrawal from alcohol liquid diet or alcohol vapor, interpreted by the authors to reflect depletion of peptide in the cell due to increased CRF release (Funk et al. 2006; Zorrilla et al. 2001). Furthermore, following the development of alcohol dependence, the CeA becomes sensitized to CRF effects, such that the ability of CRF to augment mIPSC frequency is increased and CRFR1 antagonists have a greater suppressive effect on basal inhibitory transmission in dependent vs. nondependent rats (Roberto et al. 2010). Overall, this suggests that heightened CRF signaling during acute alcohol withdrawal possibly contributes to escalated alcohol self-administration during withdrawal. During protracted withdrawal, neuroadaptations to CRF signaling in the CeA may continue to occur. Three weeks after cessation of alcohol vapor, CRF mRNA is increased in dependent rats compared to alcohol-naïve controls (Sommer et al. 2008), and after 6 weeks of alcohol withdrawal, alcohol-dependent rats have increased CRF tissue levels compared to alcohol-naïve rats (Zorrilla et al. 2001). Overall, this suggests that chronic alcohol effects on CRF signaling in the CeA last long after alcohol exposure is terminated.

A large population of CRF neurons also exists within the BNST, and these neurons are also affected by alcohol dependence and withdrawal. Like what is observed in the CeA, there is a trend toward a decrease in CRF-ir in alcohol-dependent animals compared to nondependent controls (Funk et al. 2006). During alcohol withdrawal, there is an increase in extracellular CRF in the BNST (Olive et al. 2002). Interestingly, this putative increase in CRF release in the BNST is normalized by oral alcohol consumption (Olive et al. 2002). Recent work suggests

that during acute withdrawal, increases in extracellular CRF in BNST activate glutamatergic neurons that project from the BNST to the VTA (Silberman et al. 2013). This is another example of how CRF signaling in brain stress regions impacts brain reward signaling after chronic alcohol exposure, which may be important for mediating escalated alcohol drinking, which may in turn normalize dysregulated CRF signaling.

Similar to binge drinking, chronic intermittent alcohol vapor exposure alters CRF expression in the mPFC. Twenty-four hours into withdrawal from chronic alcohol vapor, there are more CRF-positive cells in the mPFC; however, these cells are not more highly activated, as measured by c-fos, despite a large increase in c-fos expression in mPFC GABAergic interneurons at the same time point (George et al. 2012). Overall, this suggests that chronic alcohol dysregulates CRF within the mPFC (George et al. 2012), but a functional role for mPFC CRF signaling in alcohol dependence-related behavior has not yet been established.

3.3.2 Ucn1

Chronic alcohol does not alter Ucn1 levels in the EWcp, but does affect Ucn1 circuit function (Weitemier and Ryabinin 2005); more specifically, chronic alcohol decreases the number of Ucn1 fibers projecting to the lateral septum and the DRN (Weitemier and Ryabinin 2005). This change is associated with increased CRFR2 binding in the lateral septum and DRN after chronic alcohol (Weitemier and Ryabinin 2005), suggesting a potential role for this circuit in mediating behavioral change after chronic alcohol exposure. More work is needed to understand precisely how alcohol dependence and withdrawal affect Ucn1 levels and circuit function and the impact of those changes on behavior.

3.3.3 CRF Receptors

Chronic alcohol alters CRF receptor expression and function in specific ways according to receptor subtype and brain region. Acute alcohol increases CRFR1 hnRNA in the PVN (Lee et al. 2001), but this increase is blunted in rats with a history of chronic alcohol, and this blunting effect, which lasts at least 7 days, may be important for mediating altered HPA activity during alcohol dependence (Lee et al. 2001). During acute withdrawal from alcohol vapor, CRFR1 mRNA trends to be unregulated in the CeA, but not in the BLA or NAc (Roberto et al. 2010). Functionally, this same study demonstrated increased CRFR1 enhancement of GABA release in the CeA during acute withdrawal (Roberto et al. 2010). Two-week withdrawal from alcohol vapor produces robust increases in CRFR1 mRNA in the CeA, but not the MeA or BLA of mice (Eisenhardt et al. 2015). Interestingly, in rats the opposite is true, with 3 weeks of withdrawal from alcohol vapor increasing CRFR1 gene expression in the BLA and MeA, but not the CeA or BNST (Sommer et al. 2008). The reason for the difference in CRFR1 gene expression in mice compared to rats is unclear but may be attributable to the amount of time between ethanol vapor and sacrifice in those studies.

Chronic alcohol affects CRFR2 gene expression in a brain region-dependent manner. During protracted withdrawal from alcohol vapor, CRFR2 gene expression

is downregulated in the BLA of rats (Sommer et al. 2008); however, chronic alcohol exposure does not significantly change CRFR2 gene expression in the CeA, MeA, or BNST (Eisenhardt et al. 2015; Sommer et al. 2008). In mice, chronic alcohol injections increase CRFR2 binding in the DRN, a region that receives strong Ucn1 inputs from the EWcp (Weitemier and Ryabinin 2005). Also, alcohol-preferring rats show decreased CRFR2 expression compared to alcohol-non-preferring rats in the hypothalamus, amygdala, and caudate putamen. Finally, alcohol-preferring rats (iP rats) have a polymorphism in the CRFR2 gene that is associated with lower CRFR2 binding in the amygdala, which may contribute to the increased alcohol drinking behavior observed in those animals (Yong et al. 2014).

Overall, alcohol dependence and withdrawal lead to increases in extended amygdala CRF signaling (Table 1) that are hypothesized to functionally contribute to dependence-induced increases in alcohol consumption and negative affect (discussed below). In addition, this is associated with potentially decreased signaling of Ucn1 in the lateral septum and DRN, suggesting a potentially dysregulated balance between CRFR1 and CRFR2 signaling. It is not yet clear how alcohol dependence changes CRF signaling in brain reward regions, especially in the VTA, which exhibits transient increases in CRF signaling after binge-like alcohol consumption. In addition, little work has been done to examine how alcohol dependence and withdrawal affect CRF-BP levels, CRF-BP function, and Ucn2/3-CRFR2 signaling.

4 Brain Region-Specific CRFR1 and CRFR2 Effects on Alcohol-Related Outcomes

4.1 Alcohol Effects at the Synapse

The relationship between brain CRF signaling and alcohol effects on neurotransmission has been extensively studied in the extended amygdala. Within the CeA, acute alcohol increases GABAergic transmission, and antagonizing CRFR1 blocks this effect (Nie et al. 2004, 2009; Roberto et al. 2010). In alcohol-dependent rats, although there is not tolerance to the effect of acute alcohol on GABA release, antagonizing CRFR1 more effectively reduces basal and alcohol-induced increases in GABAergic transmission in CeA (Roberto et al. 2010). In addition, chronic CRFR1 blockade in CeA blocks the transition to dependence-induced escalation of alcohol drinking (Roberto et al. 2010). Overall, these findings suggest that the transition to alcohol dependence is characterized in part by alcohol-induced neuroadaptations in CeA CRF-CRFR1 signaling and that these neuroadaptations mediate some of the behavioral changes seen during and following the transition to alcohol dependence.

In the BNST, protracted withdrawal from alcohol vapor impairs long-term potentiation (LTP) induction (Francesconi et al. 2009). This impairment appears to be mediated by CRF, as repeated systemic administration of CRFR1 antagonist during withdrawal abolished the impairment in LTP induction, and repeated but

not acute CRF administration mimicked the withdrawal-induced impairment (Francesconi et al. 2009). Repeated administration of CRFR2 agonist during withdrawal had no effect on LTP induction in alcohol-dependent rats during protracted withdrawal (Francesconi et al. 2009). Overall, these results suggest that alcohol withdrawal produces neuroadaptations in BNST CRF-CRFR1 signaling that may be important for mediating behavioral change.

4.2 Alcohol Reinforcement

The role of CRF in the positive reinforcing effects of alcohol has not been extensively studied, but brain CRF signaling alters the rewarding properties of alcohol, as measured by alcohol conditioned place preference (CPP). Conditioned place preference is an indirect way of testing the reinforcing properties of a drug by pairing the drug with specific external stimuli; animals express their preference or aversion for drug-paired stimuli by approaching them or avoiding them, respectively, and this behavioral readout is thought to reflect drug reward or aversion. Although CRF is not often tested for its role in the positive reinforcing effects of alcohol (and other drugs), CRF-deficient mice fail to show an alcohol CPP at 2 g/kg alcohol but do exhibit alcohol CPP at 3 g/kg alcohol (Olive et al. 2003). Experimental inhibition of glucocorticoid synthesis or secretion does not alter the acquisition or expression of an alcohol CPP, suggesting that the change in alcohol CPP in CRF KO rats is due to extra-hypothalamic processes (Chester and Cunningham 1998). Similar to CRF KO mice, Ucn1 and CRFR2 KO mice fail to show alcohol CPP at 2 g/kg alcohol (Giardino et al. 2011); however, a higher dose of alcohol was not tested, so it remains to be seen if alterations in alcohol CPP in these knockout strains is dose-dependent, similar to what is seen in CRF-deficient mice. These whole-brain knockout mice suggest that brain CRF signaling is involved in alcohol reinforcement, but the brain region-specific roles of CRF, the Ucns, and their receptors have not been tested. Furthermore, it is unknown how CRF modulation of the positive reinforcing effects of alcohol may change during the transition to dependence.

The negative reinforcing effects of alcohol are often tested by examining negative affective symptoms and increased alcohol drinking during withdrawal (discussed below). Place conditioning can also be used to assess aversion associated with alcohol and/or alcohol withdrawal. Rats show conditioned place aversion (CPA) to a chamber paired with acute withdrawal from high doses of acute alcohol (Morse et al. 2000). Similarly, injecting mice with alcohol immediately after removal from the conditioning chamber produces a CPA that is observed in wild-type and Ucn1 knockout mice (Giardino et al. 2011), suggesting that Ucn1 signaling via CRFR1 may not mediate the aversive and/or negative reinforcing effects of alcohol (i.e., perhaps this effect is mediated by CRF signaling). In support of this, rats show a conditioned place aversion to a chamber paired with CRF infused into the ventricles, the vmPFC, or the CeA (Cador et al. 1992; Itoga et al. 2016; Schreiber et al. 2017), which suggests that excess brain CRF signaling is aversive and may support the notion that CRF mediates the aversive effects of alcohol.

4.3 Alcohol Consumption

4.3.1 Low-Level Alcohol Consumption

During consumption of low alcohol quantities by a nondependent individual, the positive reinforcing effects of alcohol drive alcohol intake, and there is limited engagement of brain stress systems (Koob 2003; Koob and Le Moal 1997). As chronicity and quantity of alcohol consumption increase and withdrawals are experienced, brain stress systems are increasingly engaged and become important for mediating escalated alcohol consumption (Koob 2003). As mentioned above, although brain CRF signaling is not typically assigned a major role in mediating the positive reinforcing effects of low doses, non-binge, and nondependent levels of alcohol, brain CRF signaling may not be without a role in the maintenance of low-level alcohol drinking (see Table 2 for a summary of the effects of brain region-specific CRF system manipulations and Table 3 for a summary of Ucn system manipulations on different types of alcohol drinking).

CRF

CRF may play a role in non-escalated alcohol consumption, because CRF knockout mice drink twice as much alcohol as their wild-type counterparts (Olive et al. 2003), and CRF-overexpressing mice show reduced alcohol consumption and preference (Palmer et al. 2004). In addition, acute intraventricular CRF reduces acute alcohol drinking in mice (Bell et al. 1998). It is not clear why the direction of whole-animal and ventricular CRF effects on low-level alcohol drinking in mice is opposite to what is typically observed in procedures that engender high levels of alcohol consumption in rats and mice. Regardless, it is likely that chronically escalated alcohol consumption and/or repeated withdrawal produces neuroadaptations that fundamentally change the role of brain CRF signaling in alcohol-related behaviors.

Ucn1

Ucn1 neurons in the EWcp modulate acute low-level alcohol drinking in a concentration-dependent manner. At low alcohol concentrations (3–10% v/v), lesion of the EWcp decreases alcohol preference and consumption in a two-bottle choice continuous access paradigm (Bachtell et al. 2004), but at higher alcohol concentrations (20% v/v), lesions of the EWcp do not affect alcohol consumption (Bachtell et al. 2004). Interestingly, the role of EWcp Ucn1 neurons in alcohol drinking seems to differ from the role of the EWcp as a whole. In a two-bottle choice continuous access paradigm, Ucn1 knockout mice do not differ from wild-type controls in levels of alcohol consumption or alcohol preference of 10% (v/v) (Giardino et al. 2017). Despite being activated by moderate doses of alcohol, it does not appear that Ucn1 signaling modulates moderate alcohol consumption, and the effects of EWcp lesion on decreasing alcohol consumption in mice may be attributable to other neuropeptides and signaling pathways.

Table 2 Effects of brain region-specific CRF system manipulations on alcohol consumption

	Systemic/ICV	Intra-CeA	Intra-BNST	Intra-VTA	Intra-DRN
Effects on low-level alcohol consumption					
CRF	↑ (KO) ↓ (Overexpress; ICV)	?	?	?	?
CRFR1 antagonism	↔	↔	↔	?	?
Effects on binge-like alcohol consumption					
CRF	↓ (KO – DID)	?	↓ (Gi – BNST; DID) ↓ (Gi – BNST → VTA; DID)	↔ (Gi – VTA; DID)	?
CRFR1 antagonism	↓ (DID)	↓ (DID)	? (DID)	? (DID)	? (DID)
	↓ (Escalation model)	? (Escalation model)	? (Escalation model)	↓ (Escalation model)	↓ (Escalation model)
CRFR2 KO/antagonism	↔ (KO – DID) ↑ (KO – escalation model)	?	?	↓ (Antagonism – DID)	?
CRF-BP KO/inhibition	↔ (KO – DID)	↔ (Inhibition – DID)	?	↓ (Inhibition – DID)	?
Effects on dependence-induced increases in alcohol consumption					
CRF	No escalation	?	?	?	?
CRFR1 antagonism	↓	↓	↔	?	?

↑ increase in alcohol consumption due to manipulation, ↔ no change in alcohol consumption, ↓ decrease in alcohol consumption,
? unknown
CRF corticotropin-releasing factor, *CRF-BP* CRF-binding protein, *CRFR1* CRF receptor type 1, *CRFR2* CRF receptor type 2, *ICV* intraventricular, *CeA* central amygdala, *BNST* bed nucleus of stria terminalis, *VTA* ventral tegmental area, *DRN* dorsal raphe nucleus

Table 3 Alcohol interactions with the urocortin system

	Systemic/ICV	CeA	EWcp
Alcohol effects on Ucn1			
Low-level/acute EtOH (Ucn1)	N/A	?	↑ Activation
Binge-like alcohol (Ucn1)	N/A	?	↑ (Escalation model) mRNA
Chronic alcohol (Ucn)	N/A	?	↔ Content ↓ Fibers projecting to lateral septum and DRN
Ucn manipulation effects on alcohol consumption			
Ucn1 effect on low-level alcohol consumption	↔ (KO)	?	?
Ucn1 effect on binge-like alcohol consumption	↔ (KO – DID) ↓ (KO – escalation model)	?	?
Ucn3 effect on binge-like alcohol consumption	↓	?	?
Ucn3 effects on dependence-induced increases in alcohol consumption	↓	↓	?

↑ increase, ↔ no change, ↓ decrease,
? unknown
ICV intraventricular, *Ucn1* urocortin-1, *Ucn3* urocortin-3, *CeA* central amygdala, *EWcp* centrally projecting Edinger-Westphal nucleus

CRFR1

CRFR1 modulation of nondependent alcohol drinking is contingent on the concentration of alcohol and the amount of alcohol the animal consumes. In nondependent animals consuming alcohol at low concentrations (<20%), systemic antagonism of CRFR1 or CRFR1 knockout has no effect on alcohol consumption (Chu et al. 2007; Gehlert et al. 2007; Roberto et al. 2010; Sabino et al. 2006). In addition, when mice do not drink in a binge-like manner or reach binge levels of alcohol consumption (BACs of <40 mg/dL), systemic CRFR1 antagonism does not alter alcohol consumption (Lowery-Gionta et al. 2012; Sparta et al. 2008). Collectively, these data suggest that CRFR1 signaling does not modulate low-level alcohol consumption.

In nondependent animals, CRFR1 signaling mediates consumption of high alcohol concentrations or high quantities of alcohol. In nondependent rats, systemic CRFR1 antagonism does not affect low-level operant self-administration without induction of dependence (Funk et al. 2006; Gehlert et al. 2007; Gilpin et al. 2008). For this reason, the effects of brain region-specific CRFR1 manipulations on low-level alcohol drinking have not been extensively studied, except in control groups in alcohol dependence studies. Those studies have shown that CRFR1 antagonism in either the CeA or BNST does not affect alcohol drinking in nondependent drinkers (Finn et al. 2007; Funk et al. 2006). However, as animals drink more alcohol or the concentration of alcohol is increased, CRFR1 signaling plays a larger role in the maintenance of alcohol consumption in nondependent animals. The effects of CRFR1 antagonism on nondependent rats are sensitive to alcohol concentration, reducing alcohol consumption at 20% v/v, but not at lower concentrations, in

a continuous access drinking procedure (Cippitelli et al. 2014). In a rat model of escalating alcohol consumption, CRFR1 antagonists reduce alcohol consumption in animals that consume the highest quantities of alcohol (Simms et al. 2014). Overall, these data suggest that CRFR1 signaling is recruited as levels of alcohol intake increase over time, even in nondependent animals.

In nondependent animals drinking low quantities of alcohol, basal CRF-CRFR1 signaling does not appear to modulate alcohol consumption, but brain CRF system signaling may modulate alcohol drinking in nondependent animals consuming high alcohol concentrations and/or quantities.

4.3.2 Binge-Like Alcohol Consumption

Brain CRF signaling is increased during repeated binge-like alcohol intake, and pharmacologic manipulations of CRF signaling during binge-like alcohol consumption may alter the transition to alcohol dependence (Lowery-Gionta et al. 2012; see Table 2).

CRF

Repeated cycles of binge-like alcohol consumption increase CRF-ir in the extended amygdala, suggesting a role of CRF signaling in mediating binge-like alcohol drinking. In support of this hypothesis, whole-brain CRF knockout mice consume less alcohol over all 4 days of the DID procedure (Kaur et al. 2012). In particular, CRF neurons in the BNST that project locally and those that project out of the BNST mediate binge-like alcohol consumption (Pleil et al. 2015; Rinker et al. 2017). Chemogenetic inhibition of all BNST local and projection CRF neurons reduces binge drinking in the DID procedure (Pleil et al. 2015). BNST CRF neurons project to the VTA, a brain region critical for alcohol reward and binge-like alcohol drinking (Dabrowska et al. 2016). Indeed, specific inhibition of VTA-projecting CRF projection neurons in BNST reduces binge-like alcohol drinking (Rinker et al. 2017). Interestingly, inhibition of CRF neurons originating in VTA does not affect binge-like alcohol drinking, suggesting that BNST CRF inputs to VTA, but not local VTA CRF neurons, are important for mediating binge-like alcohol drinking in mice (Rinker et al. 2017). It also once again suggests that brain stress systems interact with brain reward systems and that this interaction may (1) increase with heavy bouts of alcohol consumption and (2) mediate the transition to alcohol dependence.

Ucn1

Ucn1 signaling may mediate excessive binge-like alcohol consumption, although its potential role is not completely understood. Whole-brain Ucn knockout mice do not show a change in alcohol consumption in the DID procedure (Kaur et al. 2012). However, in an escalating continuous access drinking procedure, whole-brain knockout of Ucn1 decreases consumption of high alcohol concentrations (40% v/v), such that knockout mice fail to reach intoxicating binge-like intake levels and exhibit lower BACs than wild-type controls (Giardino et al. 2017). This effect was alcohol-specific, as Ucn1 knockout mice did not exhibit altered sweet or bitter taste reactivity (Giardino et al. 2017). In contrast to the DID procedure, where mice

achieve binge-like alcohol consumption in a 4-day procedure without escalating concentrations, this escalating continuous access model increases the alcohol concentration from 10 to 40% v/v over the course of 12 days (Giardino et al. 2017). The difference in these studies suggests that Ucn1 contributes to escalation of alcohol intake over time resulting in binge-like levels of alcohol consumption, but may not contribute to non-escalating binge-like alcohol drinking as modeled in the DID procedure.

CRFR1

Systemic CRFR1 antagonism and whole-brain CRFR1 knockout decrease binge-like alcohol drinking in the DID model (Kaur et al. 2012; Lowery et al. 2010; Lowery-Gionta et al. 2012; Sparta et al. 2008) and in modified two-bottle choice drinking paradigms where animals reach binge-like levels of alcohol consumption (BACs >80 mg/dL; Cippitelli et al. 2014; Simms et al. 2014). As mentioned above, CRF-CRFR1 signaling is increased in CeA during repeated binge-like alcohol consumption (Lowery-Gionta et al. 2012), and antagonizing CRFR1 in the CeA reduces binge-like alcohol drinking in the DID procedure (Lowery-Gionta et al. 2012). CRFR1 antagonism in VTA also decreases binge-like alcohol drinking in an intermittent access drinking model in rats and in high-alcohol-drinking mice (Hwa et al. 2013), and CRFR1 antagonism in DRN decreases binge-like levels of alcohol drinking in mice and rats (Hwa et al. 2013). These data clearly delineate a role for CRFR1 in binge alcohol drinking, perhaps via interactions with brain DA and/or 5-HT systems.

Ucn2/3-CRFR2

The role of Ucn2/3-CRFR2 signaling in binge-like alcohol drinking depends on the brain region and the animal model of alcohol consumption. Whole-brain deletion of CRFR2 increases alcohol intake in a limited access model of alcohol consumption (Sharpe et al. 2005), but does not affect binge-like alcohol consumption in the DID model (Kaur et al. 2012). Conversely, intraventricular administration of Ucn3 decreases binge-like alcohol drinking in the DID model (Lowery et al. 2010). Overall this suggests that activation of whole-brain Ucn3-CRFR2 signaling protects against excessive alcohol drinking. Interestingly, antagonizing CRFR2 specifically in the VTA decreases binge-like alcohol drinking in mice (Albrechet-Souza et al. 2015), suggesting that VTA CRFR2 signaling may have a unique role in mediating excessive alcohol intake.

CRF-BP

Similar to CRFR2, CRF-BP effects on binge-like alcohol consumption depend on the brain region being tested. Whole-brain CRF-BP knockout in mice does not alter binge-like alcohol drinking in the DID model (Ketchesin et al. 2016). In the CeA, inhibition of CRF-BP does not affect binge-like alcohol drinking; however, as with CRFR2, inhibition of CRF-BP in the VTA decreases binge-like alcohol drinking (Albrechet-Souza et al. 2015). Furthermore, antagonizing both CRF-BP and CRFR2 decreases binge-like alcohol consumption to a greater degree than antagonizing

CRFR2 alone, suggesting that in the VTA, CRF-BP facilitates CRF signaling, potentially through an association with CRFR2 (Albrechet-Souza et al. 2015). More work needs to be done to determine how CRF-BP signaling site specifically influences binge-like alcohol consumption.

In summary, CRF signaling plays a major role in mediating binge-like alcohol consumption, especially in the VTA. CRF projections from the BNST to the VTA mediate binge-like alcohol consumption, perhaps via signaling at both CRFR1 and CRFR2. Ucn1 signaling contributes to binge-like escalation of alcohol consumption, while Ucn3 signaling may be protective against binge-like alcohol drinking behavior. Future work should determine the relative roles of CRF and Ucn signaling in mediating binge drinking and also how connections between brain stress and reward circuits mediate binge-like alcohol drinking.

4.3.3 Dependence-Induced Increases in Alcohol Consumption

Brain CRF signaling plays a key role in dependence-induced escalation of alcohol drinking. In rodent models of chronic high-dose alcohol exposure, excessive alcohol drinking during acute and protracted withdrawal is a key sign of alcohol dependence (Edwards et al. 2012; Gilpin et al. 2008) and is mediated, at least in part, by brain CRF signaling, especially in the CeA and neighboring regions.

CRF-CRFR1

Dependence-induced escalation of alcohol drinking is highly contingent on CRFR1 signaling, and CRF-CRFR1 signaling is recruited during the transition to alcohol dependence. Whole-brain CRF knockout mice do not increase alcohol intake after induction of alcohol dependence (Chu et al. 2007), suggesting that CRF is necessary for the dependence-induced escalation of alcohol drinking. Conversely, a non-specific CRFR antagonist injected into the ventricles decreases dependence-induced increases in alcohol self-administration 2 h and 5 weeks into forced abstinence (Valdez et al. 2002). In addition, chronic systemic injection of a CRFR1 antagonist during alcohol withdrawal periods blocks escalation of alcohol self-administration, relative to alcohol-dependent rats treated chronically with vehicle (Roberto et al. 2010). In this study, at least 24 h passed between CRFR1 antagonist injections and subsequent operant self-administration sessions, suggesting that CRFR1 antagonists may block neuroadaptations that normally accumulate with repeated withdrawals (Roberto et al. 2010). In addition, systemic CRFR1 antagonism decreases alcohol intake in alcohol-dependent rats during acute and protracted withdrawal (Chu et al. 2007; Funk et al. 2007; Gehlert et al. 2007; Gilpin et al. 2008; Roberto et al. 2010). The CeA is critical for mediating CRFR1 effects on escalated alcohol drinking, because antagonizing CRFR1 in the CeA, but not the BNST or nucleus accumbens shell, decreases operant alcohol self-administration in alcohol-dependent rats (Finn et al. 2007; Funk et al. 2006).

Ucn3-CRFR2

CRFR2 activation appears to have effects that are opposite of CRFR1 activation effects on dependence-induced escalation of alcohol drinking. Intraventricular

administration of Ucn3 attenuates high alcohol drinking in alcohol-dependent rats (Valdez et al. 2004). Like CRFR1, the effect appears to be mediated in the CeA, since intra-CeA administration of Ucn3 also decreases dependence-induced alcohol drinking (Funk and Koob 2007). In light of the different effects of whole-brain and VTA modulation of CRFR2 signaling on binge-like alcohol drinking, it will be interesting to see how Ucn3-CRFR2 signaling in VTA modulates escalated alcohol drinking in alcohol-dependent animals.

In summary, CRFR1 and CRFR2 signaling have opposite effects on dependence-induced escalations in alcohol consumption. CRFR1 signaling increases alcohol consumption, and CRFR2 signaling may counteract this effect. Little work has examined a role for Ucn1/2 in dependence-induced escalation of alcohol drinking, and little is known about the CRF circuits mediating escalation of alcohol drinking during alcohol dependence.

4.4 Alcohol Relapse (e.g., Reinstatement)

AUD is defined as a chronically relapsing disorder. Environmental stimuli associated with alcohol (i.e., cues) and stressful events can each elicit relapse drinking after a period of alcohol abstinence (Sinha 2001). In rodents, reinstatement of alcohol seeking can be induced by stress or an alcohol-paired cue and is typically quantified as an increase in previously extinguished alcohol responding elicited by one of these stimuli.

4.4.1 Cue-Induced Reinstatement

Reinstatement of alcohol seeking in response to a cue formerly associated with alcohol reward may or may not be mediated by brain CRF signaling. In one study, cue-induced reinstatement of alcohol seeking was not blocked by a non-specific peptide CRFR antagonist that targeted both CRFR1 and CRFR2 (Liu and Weiss 2003). But a more recent study suggests that CRFR1 might play a role in cue-induced reinstatement; that study showed that rats treated with a systemic CRFR1 antagonist respond less on an alcohol-paired lever after the presentation of an alcohol-paired cue, relative to vehicle-treated rats (Galesi et al. 2016). This effect may be mediated by hypothalamic CRF signaling, because systemic CRFR1 antagonist effects were mimicked by systemic injection of metyrapone, a glucocorticoid synthesis inhibitor (Galesi et al. 2016). More studies are needed to clarify these apparently contradictory results and to clarify the potential role for hypothalamic and extra-hypothalamic CRF signaling in cue-induced reinstatement of alcohol seeking.

4.4.2 Stress-Induced Reinstatement

Stress is a major trigger for relapse drinking in abstinent alcoholics (Sinha 2001). In animals, stress-induced reinstatement of alcohol seeking is a paradigm in which acute stress promotes alcohol seeking by increasing the frequency of a previously extinguished operant alcohol response. In rats, this is generally accomplished with footshock or with administration of yohimbine, an alpha-2 adrenergic receptor

antagonist. Footshock reliably increases responding on a previously alcohol-paired (and previously extinguished) lever, and systemic yohimbine injection mimics this effect (Le et al. 2000).

CRF signaling is intimately involved in stress-induced reinstatement of alcohol seeking. A non-specific CRFR antagonist blocks reinstatement of alcohol seeking induced by footshock in rats (Le et al. 2000; Liu and Weiss 2003). Furthermore, footshock-induced reinstatement can be mimicked by intraventricular CRF administration (Le et al. 2002). The role of CRF in stress-induced reinstatement seems primarily due to CRFR1 because systemic administration of a selective CRFR1 antagonist attenuates footshock stress- and yohimbine-induced reinstatement of alcohol seeking in rats (Gehlert et al. 2007; Le et al. 2000; Marinelli et al. 2007). In contrast to cue-induced reinstatement of alcohol self-administration, stress-induced reinstatement is likely mediated by extra-hypothalamic CRF signaling because adrenalectomy has no effect on stress-induced reinstatement or the ability of CRFR1 antagonism to decrease alcohol-seeking behavior following footshock (Le et al. 2000). Specifically, brain stem regions including the nucleus incertus (NI) and the median raphe nucleus (MRN) appear to have a prominent role in stress-induced reinstatement mediated by CRFR1. The NI is a brain region characterized by dense CRFR expression (Potter et al. 1994) and is sensitive to exogenous CRF administration (Bittencourt and Sawchenko 2000). Recently, its role in alcohol-related behaviors has begun to be explored. CRFR1 antagonism, but not CRFR2 antagonism, in the NI attenuates yohimbine-induced reinstatement of alcohol seeking in iP rats (Walker et al. 2016). Interestingly, unlike systemic CRFR1 antagonists, site-specific antagonism in the NI did not completely reverse stress-induced reinstatement of alcohol seeking, suggesting involvement of other brain regions and perhaps interaction with other neurotransmitter systems in this behavior. One such possible candidate site is the MRN, a brain region rich in serotonergic cells that express CRFR1 and CRFR2 (Chalmers et al. 1995). Intra-MRN CRF infusion mimics footshock-induced reinstatement of alcohol seeking, and CRFR antagonism in the MRN blocks increased alcohol-seeking behavior following footshock in rats, perhaps suggesting a CRF-5-HT interaction in mediating alcohol relapse (Le et al. 2002). CRF may also interact with the kappa opioid system to induce relapse to alcohol-seeking behavior, since systemic activation of kappa opioid receptors (KOR) increases alcohol-seeking behavior, and this effect is prevented by systemic injection of a CRFR1 antagonist (Funk et al. 2014). Although these results used systemic drug injection, it is tempting to speculate that the CRF-KOR interaction may occur in the extended amygdala, due to the high degree of co-localization of CRF and dynorphin in CeA and BNST (Pomrenze et al. 2015; Reyes et al. 2001), as well as CRF and dynorphin convergent effects on GABAergic transmission in CeA (Gilpin et al. 2014; Roberto et al. 2010). Emerging preliminary data suggests that CRF-KOR interactions also occur in VTA, with obvious potential implications for alcohol reward, consumption, and seeking.

Reinstatement of alcohol seeking appears to be at least partially mediated by CRFR1 signaling. Hypothalamic CRF signaling likely contributes to cue-induced reinstatement of alcohol seeking, whereas extra-hypothalamic CRF signaling, especially in the NI and MRN, likely contributes to stress-induced reinstatement of alcohol seeking. It is not known how the urocortins and/or CRFR2 may contribute to reinstatement of alcohol seeking.

4.5 Alcohol-Induced Negative Affect (e.g., Anxiety, Nociception)

Negative affect during acute withdrawal from chronic alcohol is hypothesized to promote escalation of alcohol drinking and relapse in alcohol-dependent individuals. In fact, negative affective symptoms are reported by alcohol-dependent humans to be one of the main reasons for continual drinking (Hershon 1977; Sinha 2001). In rodents, negative affect is measured as increases in anxiety-like behavior, behavioral sensitivity to stress, and increases in nociception (i.e., hyperalgesia/allodynia). Rats exposed to repeated cycles of intoxication and withdrawal exhibit increases in anxiety-like behavior that are not seen in rats continuously exposed to alcohol (Overstreet et al. 2002). Brain CRF signaling may become sensitized during repeated withdrawals and contribute to negative affect (Koob 2003). Although much work has been done examining the role of CRF, CRFR1, and CRFR2 in negative affect associated with chronic alcohol exposure, the potential role of Ucns and CRF-BP in negative affect has been less explored (these are not covered below).

4.5.1 CRF

Brain CRF signaling may be recruited during multiple withdrawals such that brain stress systems become sensitized with repeated withdrawals and contribute to negative affect in the absence of alcohol. This hypothesis is supported by the fact that intraventricular infusions of CRF can substitute for alcohol withdrawals by mimicking repeated withdrawal-induced increases in anxiety-like behavior (Overstreet et al. 2004). This CRF effect appears to be mediated by extra-hypothalamic brain regions: repeated injections of CRF into the CeA, BLA, DRN, and dorsal BNST before initiation of alcohol liquid diet increase anxiety-like behavior during subsequent alcohol withdrawals, but CRF microinjections in the PVN, ventral BNST, or CA1 of the hippocampus do not (Huang et al. 2010). This is specific to a CRF-alcohol interaction because injections of CRF before a control diet do not alter anxiety-like behavior (Huang et al. 2010). These data support the hypothesis that repeated alcohol withdrawals sensitize the brain to the effects of future alcohol withdrawals through a brain CRF signaling mechanism. In addition, non-specific CRF receptor antagonism blocks restraint stress-induced increases in anxiety-like behavior after 6 weeks of alcohol deprivation in previously alcohol-dependent rats, suggesting a role for CRF in enhanced responsiveness to stress during protracted withdrawal from alcohol (Valdez et al. 2003).

4.5.2 CRFR1

Activation of CRFR1 during withdrawal is critical for many aspects of negative affect including anxiety-like behavior, sensitization of anxiety-like behavior during repeated alcohol withdrawals, and hyperalgesia. Systemic CRFR1 antagonism before the first and second withdrawals of a multiple-withdrawal protocol prevented increases in anxiety-like behavior normally observed during subsequent alcohol withdrawals (Overstreet et al. 2007; Breese et al. 2004). Site-specific CRFR1 antagonism in the CeA, DRN, and dorsal BNST blocks CRF-induced sensitization of withdrawal anxiety-like behavior, suggesting that stress-induced sensitization during withdrawal is mediated by extra-hypothalamic brain regions (Huang et al. 2010). Alcohol-dependent animals typically exhibit withdrawal-induced increases in anxiety-like behavior that can be blocked with systemic CRFR1 antagonism or whole-brain CRFR1 knockout (Rassnick et al. 1993; Sommer et al. 2008; Timpl et al. 1998). As with dependence-induced increases in alcohol self-administration, intra-CeA CRFR1 antagonism reverses alcohol withdrawal-induced increases in anxiety-like behavior in alcohol-dependent animals (Baldwin et al. 1991; Rassnick et al. 1993). Similarly, systemic CRFR1 antagonism decreases allodynia in alcohol-dependent rats, although the specific brain regions mediating this effect have yet to be determined (Edwards et al. 2012). Overall, CRFR1 signaling in CeA is recruited during repeated withdrawals and contributes to withdrawal-induced negative affect and drives the negative reinforcing effects of alcohol.

4.5.3 CRFR2

In stark contrast to CRFR1, CRFR2 activation decreases negative affective symptoms in alcohol dependence. Intraventricular Ucn3 decreases dependence-induced anxiety-like behavior (Valdez et al. 2004). The brain region mediating Ucn3-CRFR2 signaling-induced decreases in negative affect has not yet been determined, although Ucn3-CRFR2 signaling in the DRN and dorsal BNST, brain regions implicated in withdrawal-induced sensitization of negative affect, does not significantly contribute to decreases in anxiety-like behavior induced by dependence (Huang et al. 2010). Intra-CeA Ucn3-CRFR2 manipulations have not been tested for their effects on alcohol withdrawal-related negative affective behaviors, but Ucn3-CRFR2 signaling may have a role since intra-CeA injection of a CRFR2 agonist decreases alcohol withdrawal-induced increases in alcohol self-administration (Funk and Koob 2007). More work is needed to clearly delineate the role of Ucn3-CRFR2 in mediating alcohol dependence-induced negative affect, excessive alcohol drinking, and relapse.

Similar to escalations in alcohol drinking, negative affect in the alcohol-dependent organism is mediated by CRF-CRFR1 signaling, and it is counteracted by Ucn3-CRFR2 signaling. It will be interesting to see what role if any Ucn1 and Ucn2 play in negative affect and also which brain networks contribute to the negative affect observed in alcohol-dependent animals during withdrawal.

5 Human Studies

5.1 Status of Clinical Trials

Extensive preclinical work examining the role of CRF-CRFR1 signaling in alcohol-related behavior suggests that CRFR1 antagonists may have therapeutic potential in humans with AUD, specifically to reduce excessive alcohol drinking and symptoms of withdrawal and prevent relapse in those individuals. Although numerous small-molecule CRFR1 antagonists have been developed for clinical trials, the results have been overwhelmingly negative. One of the first molecules tested, R121919, showed some promise in depression, but development of the drug was suspended due to elevations in liver enzymes (Zobel et al. 2000). Other small-molecule CRFR1 antagonists have been tested in depression, anxiety, human fear lab studies, and PTSD, but all have yielded negative results (Binneman et al. 2008; Coric et al. 2010; Dunlop et al. 2017; Grillon et al. 2015).

Two studies have examined the efficacy of CRFR1 antagonists for decreasing craving in anxious adults with AUD. The first study tested pexacerfont, an orally available, brain-penetrant CRFR1 antagonist (Kwako et al. 2015), but that study reported no effect on alcohol craving following alcohol or stress cues or following a Trier social stress test (TSST; Kwako et al. 2015). In addition, pexacerfont had no effect on measured neuroendocrine outcomes, including cortisol and ACTH levels following stress (Kwako et al. 2015). The negative results in this study were thought to be due to the binding kinetics of pexacerfont, which has a fast receptor off-rate (Fleck et al. 2012). Therefore, a follow-up study examined therapeutic potential of CRFR1 antagonism in anxious AUD females, this time using verucerfont (Schwandt et al. 2016). Verucerfont is an orally available, brain-penetrant potently selective CRFR1 antagonist with a similar structure to compounds with slow off-kinetics, suggesting increased efficacy compared to pexacerfont (Schwandt et al. 2016). Indeed, verucerfont blunted HPA axis activity during a dexamethasone-CRF challenge, suggesting that verucerfont is more active than pexacerfont (Schwandt et al. 2016). However, like pexacerfont, verucerfont failed to reduce craving after stress cues or alcohol cues (Schwandt et al. 2016). In fact, verucerfont significantly increased anxiety in the TSST (relative to placebo) without significantly affecting alcohol craving or HPA axis activation after TSST (Schwandt et al. 2016). Overall, these data suggest that antagonism of CRFR1 in anxious humans with AUD does not affect stress- or cue-induced increases in alcohol craving, despite having activity on the HPA axis. That said, the preponderance of preclinical data strongly implicates CRFR1 signaling in mediating escalated alcohol drinking and negative affective symptoms seen during early withdrawal, which were not measured in these two clinical studies. In addition, these studies may have missed the temporal window in which CRFR1 antagonists might be expected to have efficacy, because they were completed after withdrawal symptoms subsided. Therefore, CRFR1 antagonism may still be therapeutically relevant for reducing alcohol drinking or negative affective symptoms, but likely not craving, in people with AUD.

5.2 Why the CRF System Is Still Important in AUD Research

The CRF system is still important in AUD research despite negative effects in two clinical trials examining CRFR1 antagonist effects on alcohol craving. The negative results of the clinical trials may be attributable to any combination of the following: limitations of stress-induced alcohol reinstatement in animal studies relative to the human craving it models, mismatched timing of drug delivery in animal and human studies, differences between brain CRF systems in rodents versus humans, or the possibility that CRFR1 antagonists would only be therapeutically effective in an as yet unidentified subgroup of AUD patients (Spierling and Zorrilla 2017).

The two alcohol craving clinical trials described above targeted CRFR1 signaling, but preclinical literature supports a potential role for CRFR2, the Ucns, and CRF-BP in mediating various aspects of alcohol use. A recent study in nonhuman primates postulated that in primates, CRFR2 in the amygdala might play an important role in anxiety-like responses (Kalin et al. 2016). Indeed, compared to rodents, where there is limited CRFR2 expression in the CeA (Van Pett et al. 2000), primates express high density of CRFR2 in the CeA (Sanchez et al. 1999), although the functional relevance of this has not yet been determined. CRF-BP in rodents was recently shown to interact with CRFR2 in the VTA to influence binge-like alcohol drinking (Haass-Koffler et al. 2016). Therefore, a deeper understanding of the relationship between alcohol and brain CRF system plasticity and signaling, especially CRFR2 signaling and CRF-BP, may be necessary to effectively leverage the brain CRF system as a therapeutic target for reducing excessive alcohol drinking and negative affect in at least a subset of humans living with AUD.

6 Future Directions and Conclusions

Animal studies clearly implicate the brain CRF system in mediating escalated alcohol drinking and negative affect observed in rodent models of AUD. Rodent models suggest that high-dose alcohol exposure, in the form of binge-like alcohol drinking or forced high-dose alcohol exposure (i.e., that which produces alcohol dependence), dysregulates CRF signaling in hypothalamic and extra-hypothalamic brain regions. This dysregulated CRF signaling, particularly CRF-CRFR1 signaling within the extended amygdala, is hypothesized to drive excessive alcohol drinking, negative affect, and stress-induced alcohol-seeking behaviors that are associated with alcohol dependence and binge-like alcohol drinking.

Although clinical studies have demonstrated a lack of efficacy of CRFR1 antagonists in decreasing craving in anxious adults with AUD, these studies don't entirely rule out other clinical endpoints (e.g., alcohol consumption) or other components of the brain CRF system as potential therapeutic targets for treating aspects of AUD. In addition, hypothalamic CRF signaling and the HPA axis may be of more importance in AUD than the preclinical literature suggests. In a recent study, glucocorticoid receptor antagonists decreased alcohol craving and alcohol drinking in treatment-seeking individuals with AUD (Vendruscolo et al. 2015).

Advances in basic science research technology, including optogenetics, chemogenetics, and transgenic rodent lines, allow for circuit-specific modulation of brain CRF signaling that will greatly enhance our understanding of how, where, and when brain CRF signaling modulates escalated alcohol drinking, relapse-like behavior, and negative affect. In addition, future work should examine the potential roles of CRFR2, Ucns, and CRF-BP in binge-like alcohol drinking, negative affective states associated with alcohol withdrawal, and relapse, both in rodent models and in primate models, to maximize the translational value of this work. The investigation of brain CRF system signaling remains important not only for potential therapeutic benefits but also in the investigation of CRF receptors as gatekeepers on the function of brain circuits impacted by alcohol and drugs and important for various behaviors, many of which extend beyond the addiction field (e.g., stress, fear, and pain).

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Contribution of Dynorphin and Orexin Neuropeptide Systems to the Motivational Effects of Alcohol

Rachel I. Anderson, David E. Moorman, and Howard C. Becker

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R. I. Anderson
Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina, Charleston, SC, USA

Science and Technology Policy Fellowships, American Association for the Advancement of Science, Washington, DC, USA

D. E. Moorman
Department of Psychological and Brain Sciences, Neuroscience and Behavior Graduate Program, University of Massachusetts Amherst, Amherst, MA, USA

H. C. Becker (✉)
Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina, Charleston, SC, USA

Charleston Alcohol Research Center, Medical University of South Carolina, Charleston, SC, USA

Department of Neuroscience, Medical University of South Carolina, Charleston, SC, USA

Department of Veterans Affairs, Ralph H. Johnson VA Medical Center, Charleston, SC, USA
e-mail: beckerh@musc.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_100

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Abstract

Understanding the neural systems that drive alcohol motivation and are disrupted in alcohol use disorders is of critical importance in developing novel treatments. The dynorphin and orexin/hypocretin neuropeptide systems are particularly relevant with respect to alcohol use and misuse. Both systems are strongly associated with alcohol-seeking behaviors, particularly in cases of high levels of alcohol use as seen in dependence. Furthermore, both systems also play a role in stress and anxiety, indicating that disruption of these systems may underlie long-term homeostatic dysregulation seen in alcohol use disorders. These systems are also closely interrelated with one another – dynorphin/kappa opioid receptors and orexin/hypocretin receptors are found in similar regions and hypocretin/orexin neurons also express dynorphin – suggesting that these two systems may work together in the regulation of alcohol seeking and may be mutually disrupted in alcohol use disorders. This chapter reviews studies demonstrating a role for each of these systems in motivated behavior, with a focus on their roles in regulating alcohol-seeking and self-administration behaviors. Consideration is also given to evidence indicating that these neuropeptide systems may be viable targets for the development of potential treatments for alcohol use disorders.

Keywords

Alcohol · Dynorphin · Ethanol · Hypocretin · Kappa opioid receptor · Orexin

1 Neuropeptides: Dynorphin and Orexin

1.1 Introduction

The dynorphin (DYN) and hypocretin/orexin (ORX) neuropeptide systems play critical roles in regulating appetitively and aversively motivated behaviors. Activation of both systems is associated with arousal, stress, and reward motivation. Both systems are also implicated in psychiatric diseases such as anxiety, depression, and addiction. In particular, DYN and ORX have been demonstrated to be major contributors to alcohol use and potentially misuse and dependence. In this chapter we first discuss the overarching roles of these systems in reward- and aversion-related behaviors followed by a consideration of their roles in alcohol use and dependence. In some cases these peptides are co-expressed,

raising questions about their separate versus overlapping roles in the motivational effects of alcohol. We end with a discussion of the potential interaction between these systems and future studies that could address their unique or shared contributions to alcohol consumption and alcohol use disorders.

2 Dynorphin/Kappa Opioid System and Roles in Pharmacological and Motivational Effects of Alcohol

Dynorphins are highly potent endogenous opioids that bind preferentially to kappa opioid receptors with relatively little affinity for mu and delta opioid receptor subtypes. The precursor protein prodynorphin can be cleaved to form numerous active peptides including dynorphin A, dynorphin B, big dynorphin, α and β neoendorphins, and leumorphin (Chavkin 2013). Kappa opioid receptors are G protein-coupled receptors that are distributed widely throughout the central nervous system. When activated, KORs typically couple to inhibitory G proteins and exert their effects through multiple signal transduction pathways (Bruchas and Chavkin 2010). However, lower ligand concentrations have been reported to provoke coupling to G_s proteins, initiating stimulatory signaling cascades (Crain and Shen 2000).

2.1 Dynorphin/Kappa Opioid Receptor (DYN/KOR) System Anatomy

The anatomical distribution of DYN and KOR expression in brain regions associated with reward and stress enable this neuropeptide system to contribute to addiction and mood disorders. Dynorphin immunoreactivity has been observed throughout the cortex, nucleus accumbens (NAc), striatum, caudate-putamen, lateral division of the central nucleus of the amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, multiple hypothalamic nuclei, periacqueductal gray, and numerous brainstem nuclei (Khachaturian et al. 1982; Fallon and Leslie 1986). Assessments of KOR mRNA in the human brain have revealed high expression in prefrontal cortex (PFC; particularly in deep layers), NAc, caudate-putamen, dentate gyrus of the hippocampus, thalamus, hypothalamus, amygdala, ventral tegmental area (VTA), and multiple brainstem nuclei (Peckys and Landwehrmeyer 1999; Simonin et al. 1995). Similarly, a study of KOR mRNA and KOR binding density in the rat brain reported co-expression (suggesting local receptor synthesis) in multiple brain regions including the NAc, caudate-putamen, olfactory tubercle, BNST, paraventricular nucleus of the hypothalamus (PVN), amygdala, periaqueductal gray, raphe nucleus, locus coeruleus, and nucleus of the solitary tract (Mansour et al. 1995). Within the VTA, however, few KOR binding sites are detected but KOR mRNA is highly expressed, suggesting that KORs are likely produced in this region and transported to the NAc (Mansour et al. 1995). Notably, species differences have been observed when comparing KOR expression in humans and rodents: KORs are more widely expressed in the human brain, particularly in the cortex, hippocampus, and thalamus (Peckys and Landwehrmeyer 1999).

2.2 KOR Pharmacology and Signaling

Although pharmacological evidence has suggested the existence of multiple KOR subtypes, only one KOR has been cloned (reviewed by Bruijnzeel 2009; Dietis et al. 2011). Agonist binding at these receptors can initiate the dissociation of G $\beta\gamma$ from Gα subunits or directly interact with β arrestins (Bruchas and Chavkin 2010). Consequently, KOR activation can result in stimulation of a variety of signaling cascades (including ERK 1/2, p38 MAPK, and JNK) that depend on the nature of the ligand. The β arrestin recruitment of the p38 MAPK cascade has been implicated in dysphoric effects of KOR agonism and stress (Bruchas et al. 2007). Synthesis of ligands that favor a particular signaling pathway (biased agonism) is an emerging trend in drug development, and recent efforts have aimed to develop KOR ligands that favor G-protein coupled signaling rather than β arrestin in order to diminish aversive effects in favor of therapeutic effects (Lovell et al. 2015; Zhou et al. 2013).

Kappa opioid receptors are expressed throughout the brain, with presynaptic expression enabling modulation of neurotransmission in numerous brain regions associated with drug and alcohol reward. For example, KORs located on glutamatergic projections to the PFC, NAc, dorsal striatum, BNST, and VTA inhibit signaling when activated (Hjelmstad and Fields 2001; Margolis et al. 2005; Tejeda et al. 2013; Atwood et al. 2014; Crowley et al. 2016). Similarly, stimulation of KORs expressed on dopaminergic projections to amygdala, NAc, and PFC and on GABAergic projections to amygdala, NAc, VTA, and BNST also results in decreased transmission (Ford et al. 2007; Margolis et al. 2003, 2006, 2008; Hjelmstad and Fields 2003; Li et al. 2012). KORs located on projections to multiple brain regions can also influence serotonergic and noradrenergic signaling (Berger et al. 2006; Land et al. 2009).

2.3 DYN/KOR System and Motivational Behaviors

Whereas activation of mu opioid receptors stimulates dopamine release in the NAc, activation of KORs results in reduced dopamine release in this brain region (Di Chiara and Imperato 1988). Thus, in contrast to euphoric effects that characterize mu opioid receptor activation, effects of KOR activation are largely aversive or dysphoric. For example, studies in rodents have shown that KOR activation results in conditioned taste and place aversion, decreased reward sensitivity (i.e., increased reward thresholds in intracranial self-stimulation procedures), and increased depressive-like and anxiety-like behaviors (Mucha and Herz 1985; Todtenkopf et al. 2004; Mague et al. 2003; Bruchas et al. 2009; Valdez and Harshberger 2012). Similarly, administration of KOR agonists to humans has been reported to produce aversive effects including anxiety, racing thoughts, agitation, hallucinations, confusion, sedation, and dysphoria (Pfeiffer et al. 1986; Rimoy et al. 1994; Walsh et al. 2001).

KOR activation activates the hypothalamic-pituitary-adrenocortical (HPA) axis and stimulates glucocorticoid release (Iyengar et al. 1986; Wittmann et al. 2009). Likewise, stress exposure activates and upregulates the KOR system, and DYN signaling through KORs has been implicated in the aversive effects of stress (Land

et al. 2008). On the other hand, KOR antagonists have been shown to reduce both anxiety-like and depressive-like behavior in addition to blocking the dysphoric effects of stressor exposure (Knoll et al. 2007; Carr and Lucki 2010; Land et al. 2008; Mague et al. 2003). The DYN/KOR system likely facilitates stress-related signaling through interactions with the corticotropin-releasing factor (CRF) system (Land et al. 2008; Van't Veer and Carlezon 2013). Evidence for co-localization of DYN and CRF has been observed within neurons of the central nucleus of the amygdala (CeA), PVN, and locus coeruleus (Marchant et al. 2007; Roth et al. 1983; Kreibich et al. 2008).

2.4 Alcohol and the DYN/KOR System

Interest in DYN/KOR modulation of alcohol consumption dates back to the late 1980s, and appears to have stemmed from a body of evidence that established a role for this neuropeptide system in ingestive behaviors (Sandi et al. 1988; Morley and Levine 1983). Since that time, research efforts have expanded to assess effects of alcohol on DYN/KOR expression and function, as well as effects of the DYN/KOR system on alcohol's rewarding and motivational effects (Anderson and Becker 2017).

2.4.1 Effects of Alcohol Exposure on DYN/KOR Expression and Function in Brain

Both acute and chronic alcohol exposures produce adaptations in the DYN/KOR system, typically reflected by an upregulation of expression and activity. For example, microdialysis and radioimmunoassay studies have revealed that, following acute systemic delivery of alcohol, dynorphin levels are increased in the NAc, CeA, VTA, and PVN (Marinelli et al. 2006; Lam et al. 2008; Jarjour et al. 2009; Chang et al. 2007). Elevated prodynorphin or dynorphin mRNA expression also has been observed in the amygdala, PFC, and PVN following acute alcohol administration (D'Addario et al. 2013; Chang et al. 2007). DYN-B expression was elevated in the NAc following repeated alcohol administration (Lindholm et al. 2000), although in another report, a similar alcohol exposure regimen resulted in decreased KOR mRNA expression in the NAc and VTA (Rosin et al. 1999). Chronic alcohol consumption has been shown to increase DYN mRNA and peptide levels in the PVN and prodynorphin levels in the NAc (Chang et al. 2007; Przewlocka et al. 1997). Adaptations in the DYN/KOR system also occur during alcohol withdrawal, with upregulated KOR signaling and DYN peptide expression observed in the CeA (Kissler et al. 2014).

2.4.2 Effects of KOR Activation and Blockade on Alcohol-Related Behaviors

A number of preclinical studies have examined the effects of KOR agonists and antagonists on alcohol-related behavior. Research examining effects of KOR ligands on home-cage alcohol consumption has yielded variable results in rats, with studies reporting increases, decreases, and no change in alcohol intake following both systemic KOR activation and blockade (Sandi et al. 1988, 1990; Nestby et al. 1999;

Holter et al. 2000; Lindholm et al. 2001; Mitchell et al. 2005; Morales et al. 2014; Rorick-Kehn et al. 2014). These discrepant findings are likely due to differences in experimental parameters, including sex, strain, drug dose and timing of administration, stress experience, and history of ethanol exposure (Anderson and Becker 2017). In alcohol-preferring C57BL/6J mice, however, several reports have replicated the finding that the KOR agonist U50,488 increases alcohol intake (Sperling et al. 2010; Rose et al. 2016; Anderson et al. 2016). Likewise, KOR antagonism has been consistently reported to decrease home-cage alcohol consumption in C57BL/6J mice, though this effect is typically observed only when intake is elevated above a basal level following induction of alcohol dependence or stress exposure (Sperling et al. 2010; Rose et al. 2016; Anderson et al. 2016). Only a few studies have examined the effects of KOR activation in specific brain regions on home-cage ethanol consumption. Administration of U50,488 reduced intake in rats when infused into the lateral hypothalamus or the PVN (Chen et al. 2013; Barson et al. 2010), but had no effect in the NAc or ventral pallidum (Barson et al. 2009; Kemppainen et al. 2012).

Studies using operant self-administration procedures in rats have reported that KOR agonists reduce alcohol self-administration, suggesting that KOR activation opposes the rewarding effects of alcohol (Holter et al. 2000; Henderson-Redmond and Czachowski 2014). KOR agonists are also consistently reported to induce reinstatement of alcohol-seeking behavior, an effect interpreted as a stress-like effect of KOR activation (Harshberger et al. 2016; Funk et al. 2014; Le et al. 2017). Conversely, KOR blockade has been shown to attenuate cue-induced reinstatement and reinstatement induced by pharmacological stressors (Berger et al. 2013; Schank et al. 2012; Funk et al. 2014). Both systemic and site-specific (NAc, BNST, CeA) administrations of the KOR antagonist nor-BNI have been shown to attenuate elevated self-administration in alcohol-dependent rats while not influencing responding in nondependent rats (Walker and Koob 2008; Walker et al. 2011; Nealey et al. 2011; Kissler et al. 2014; Erikson and Walker 2016).

Several reports have observed altered alcohol-induced conditioned place preference following KOR activation, suggesting that KOR signaling can influence the conditioned motivational effects of alcohol. However, the direction of the effect appears to be related to the timing of agonist administration. Specifically, administration of a KOR agonist shortly before alcohol treatment blocked the development of alcohol conditioned place preference whereas administration of the same agonist 90 min before alcohol conditioning sessions resulted in a potentiation of alcohol conditioned place preference (Logrip et al. 2009; Sperling et al. 2010). Although studies with cocaine have revealed time-dependent effects of KOR agonist administration on cocaine-induced dopamine release in the NAc (Ehrich et al. 2014; Chartoff et al. 2016), at present, it is unclear how dose of the KOR agonist (U50,488) and/or the timing of its administration in relation to alcohol intoxication influences the outcome of these conditioning studies. Interestingly, several reports suggest that KOR blockade has no effect on alcohol's conditioned motivational properties. For instance, nor-BNI did not alter expression of alcohol conditioned place preference or taste aversion under standard testing conditions (Sperling et al. 2010; Roma et al. 2008; Anderson et al. 2013; Nguyen et al. 2012).

A large literature has provided evidence that stress activates the DYN/KOR system and that increased DYN/KOR activity plays an important role in mediating behavioral responses to various stress events (Crowley and Kash 2015; Knoll and Carlezon 2010; Van't Veer and Carlezon 2013). Despite an established role for this neuropeptide system in stress-related behavior, relatively few studies have examined the influence of DYN/KOR activity in mediating the interaction between stress and alcohol reward (Becker 2017). Although these studies provide evidence to indicate such involvement, the results have not been consistent. For example, pretreatment with the KOR antagonist nor-BNI was reported to block stress-induced potentiation of alcohol conditioned place preference in mice whereas a study in rats reported that nor-BNI administration further enhanced the effects of stress on alcohol conditioned place preference (Sperling et al. 2010; Matsuzawa et al. 1999). Evidence suggests that KOR modulation of alcohol consumption is also influenced by stress conditions. Mice defeated in multiple social interactions consumed more alcohol than victorious mice, an effect that was further enhanced by administration of the KOR agonist U50,488 (Kudryavtseva et al. 2006). In another report, stress-enhanced consumption in alcohol-dependent mice was blocked by the KOR antagonist LY2444296 (Anderson et al. 2016). Similarly, a study that observed elevated alcohol consumption in adult rats exposed to isolation stress throughout adolescence found that nor-BNI administration reversed this effect (Karkhanis et al. 2016a). The same report demonstrated enhanced sensitivity to KOR agonist-induced suppression of dopamine release in the NAc of rats reared in isolation, suggesting long-lasting adaptations of the KOR system following stressful experiences (Karkhanis et al. 2016a). Taken together, a growing body of literature demonstrates that pharmacological manipulation of KORs influences the motivational effects of alcohol. This includes alcohol self-administration as well as the conditioned rewarding effects of alcohol. A host of variables, including dose, timing of drug administration, and stress experience, likely accounts for differences in outcomes. Future studies will be needed to tease apart these important variables, on both mechanistic and behavioral levels.

2.5 Brain Circuitry Analyses of DYN/KOR System Involvement in Alcohol Actions

Substantial evidence indicates that the NAc is an important site where KOR activity modulates alcohol-induced dopamine release. Indeed, systemic administration of alcohol has been shown to provoke DYN release in the NAc (Marinelli et al. 2006), and pharmacological activation of KORs has been reported to reduce alcohol-evoked dopamine release in this brain region (Lindholm et al. 2007). Although the agonist effect was independent of alcohol exposure history, KOR antagonism increased alcohol-evoked dopamine release in rats with a history of repeated alcohol treatment, but not in saline-injected controls (Lindholm et al. 2007). Additional evidence indicates that chronic alcohol exposure results in increased sensitivity of KORs in the NAc. That is, KOR agonist-induced suppression of dopamine release (measured using fast-scan cyclic voltammetry) was more pronounced in subjects exposed to chronic alcohol (Rose et al.

2016; Karkhanis et al. 2016b; Siciliano et al. 2015). These adaptations may explain why blockade of KORs in the NAc shell has been shown to selectively reduce escalated consumption in alcohol-dependent rats (Nealey et al. 2011). Interestingly, another study demonstrated that within the NAc shell, subpopulations of DYN neurons mediate reward and aversion (Al-Hasani et al. 2015). The effects of alcohol on these subpopulations have yet to be examined and are worthy of future study.

Alcohol administration also results in DYN release in the CeA (Lam et al. 2008). Induction of alcohol dependence via repeated alcohol vapor inhalation has been shown to increase both DYN peptide expression and KOR signaling within the CeA (Kissler et al. 2014). Accordingly, site-specific administration of the KOR antagonist nor-BNI into this area resulted in reduced alcohol consumption in dependent rats, but not their nondependent counterparts (Kissler et al. 2014). KOR ligands have also been reported to influence the effects of alcohol on GABAergic transmission within the CeA (Kang-Park et al. 2013; Gilpin et al. 2014).

Recent and ongoing research continues to illuminate mechanisms of DYN modulation of neural signaling in other brain regions sensitive to alcohol, although interactions with alcohol actions have not yet been well characterized. For example, KORs modulate neurotransmission within multiple projections to the BNST, a brain region implicated in alcohol seeking that shows stress-induced plasticity (Conrad et al. 2012; Pina et al. 2015). Activation of KORs on projections from the central amygdala to the BNST inhibits GABAergic transmission (Li et al. 2012), while stimulation of KORs on projections from the basolateral amygdala also inhibits glutamate transmission in the BNST (Crowley et al. 2016). Blockade of KORs in the BNST eliminates KOR agonist-induced reinstatement of operant alcohol self-administration, suggesting a role for KOR signaling in stress modulation of alcohol reward (Le et al. 2017).

The PFC may be another area where the DYN/KOR system interacts with alcohol. KORs modulate neurotransmission in the PFC, and this brain region is implicated in the role of stress in the transition to alcohol dependence (Margolis et al. 2006; Tejeda et al. 2013, 2015; Lu and Richardson 2014; Rodberg et al. 2017). Chronic alcohol exposure increases prodynorphin expression in the PFC, and a comparison of postmortem human brain tissue in alcoholic and control subjects revealed greater expression of prodynorphin and KOR mRNA in the dorsolateral PFC and orbitofrontal cortex (Bazov et al. 2013; D'Addario et al. 2013).

3 Orexin/Hypocretin Receptor System and Roles in Pharmacological and Motivational Effects of Alcohol

In contrast to the DYN/KOR system, the orexin/hypocretin (ORX) system has been less-extensively studied in the context of alcohol use/misuse. Nevertheless, in the past 10 years, interest in the role ORX plays in mediating various alcohol actions has steadily grown. There is also recognition of an intriguing overlap between ORX and DYN neuropeptides, in part due to co-localization of both peptides in the same neurons as well as the fact that both systems play fundamental roles in stress and motivation, particularly for alcohol. These and other relationships between these two

neuropeptides will be considered further after a discussion of the ORX system and its role in alcohol use and dependence.

3.1 Orexin/Hypocretin Peptide/Receptor System Anatomy

The ORX system is made up of a population of neurons located exclusively in the tuberal hypothalamus, in a region typically referred to as the lateral hypothalamic area. First discovered in rodents in 1998 where it was termed orexin (Sakurai et al. 1998) and hypocretin (de Lecea et al. 1998) by different research groups, this relatively restricted population of neurons influences a wide range of behaviors. There are approximately 70,000 ORX neurons in humans and approximately 3,000 in rats (Peyron et al. 1998; Nambu et al. 1999), but these neurons project widely across the central nervous system. ORX neurons are defined by the expression of the protein precursor prepro-orexin (preprohypocretin), which is cleaved into two active peptides: the 33 amino acid orexin-A (ORX-A), also known as hypocretin-1 (HCRT-1), and the 28 amino acid orexin-B (ORX-B), also known as hypocretin-2 (HCRT-2). There are two ORX receptors (OXRs), OX1R (HCRTR1) and OX2R (HCRTR2), which exhibit differential selectivity for ORX-A vs. ORX-B, and activation of these receptors mediates numerous physiological functions.

ORX neurons residing in the hypothalamus project widely across the extent of the brain and spinal cord (Peyron et al. 1998; Date et al. 1999; Nambu et al. 1999; van den Pol 1999; Nixon and Smale 2007). Among the many projection targets, the ORX system strongly innervates a number of regions associated with motivation for natural and drug rewards, as well as those associated with emotional regulation, including stress. This includes projection sites with dense ORX terminal expression such as the noradrenergic locus coeruleus, dopaminergic midbrain areas such as the VTA and substantia nigra, the serotonergic raphe nuclei, the cholinergic laterodorsal and peducopontine nuclei, BNST, CeA, a number of thalamic nuclei, and more local projections among numerous nuclei across the extent of the hypothalamus (Peyron et al. 1998; Date et al. 1999; Nambu et al. 1999; Nixon and Smale 2007). Other brain areas, particularly those involved in regulating sleep and arousal, such as the noradrenergic locus coeruleus and the histaminergic tuberomammilary nucleus, are also heavily innervated, demonstrating an additional important role for this system in regulating arousal states (Sakurai 2007). As reviewed below, ORX activity in a number of these targets has been shown to have profound effects on reward seeking, including alcohol seeking, and emotional arousal and regulation.

3.2 ORX Receptor Pharmacology and Signaling

As noted above, ORX peptides exert their effects through two receptor subtypes: the orexin-1 and orexin-2 receptors (OX1R and OX2R, also referred to as HcrtR1 and HcrtR2). OX1R binds ORX-A with high affinity and ORX-B with low affinity, whereas OX2R binds equally to both peptide subtypes. OX1R couples with Gq proteins, resulting

in excitation via nonselective cation channels, voltage-gated Ca2+ channels, Na+/Ca2+ exchange, and inhibition of K+ channels, whereas OX2R is Gq- and/or Gi/Go-protein coupled, resulting in more complex signaling outcomes depending on G protein profile (Tang et al. 2008; Kukkonen and Leonard 2014; Sakurai 2014; Kukkonen 2017; Schone and Burdakov 2017). OXRs can also have indirect effects via regulation of NMDA receptors, or via altering presynaptic glutamate or GABA release (Li et al. 2002; Liu et al. 2002; Borgland et al. 2006, 2008; Baimel and Borgland 2012). OX1Rs and OX2Rs are widely distributed across the brain. Within hypothalamic nuclei there is some overlap between receptor subtypes, but in many other regions the two receptor subtypes appear to exhibit complementary expression, with either exclusive or biased expression of one subtype over another (Trivedi et al. 1998; Hervieu et al. 2001; Marcus et al. 2001; Cluderay et al. 2002). For example, OX1R expression is more predominant in areas such as PFC, amygdala nuclei, CA1 and CA2 (but not CA3) hippocampal regions, laterodorsal tegmental area, and locus coeruleus. In other areas, such as the VTA, as well as a number of thalamic nuclei, receptor distribution is approximately equivalent, and OX2Rs predominate in other regions, notably in a number of hypothalamic nuclei, brainstem nuclei, lateral habenula, and other regions. This semi-differential distribution has led some investigators to propose that signaling through the OX1R is more related to emotional and motivational control whereas OX2R signaling conveys the influence of the ORX system on arousal (Sakurai 2014). However, this dichotomy is far from exclusive. For example, the locus coeruleus, which regulates sleep and arousal, exhibits extremely dense expression of OX1Rs. In contrast, the shell of the NAc, an area closely associated with appetitively motivated behaviors, preferentially expresses OX2Rs (Trivedi et al. 1998; Hervieu et al. 2001; Marcus et al. 2001; Cluderay et al. 2002). Thus, attributing specific behavioral effects to actions at OX1Rs vs. OX2Rs remains a challenge, and this includes ORX-mediated effects on alcohol-related behaviors.

The effects of ORX on postsynaptic neurons are largely excitatory, via mechanisms noted above. The extensive projections of the ORX system, particularly to areas such as the VTA, locus coeruleus, BNST, and multiple thalamic, hypothalamic, and amygdalar nuclei, indicate that these neurons produce a potent excitatory drive on a number of regions influential in arousal, emotion, and motivation. For example, ORX release in the VTA produces excitatory plasticity and increases firing of dopamine neurons in vitro and in vivo (Borgland et al. 2006; Korotkova et al. 2006; Muschamp et al. 2007; Moorman and Aston-Jones 2010), as well as elevating dopamine release in VTA targets such as the PFC and NAc (Vittoz and Berridge 2006; Calipari and Espana 2012; Prince et al. 2015). ORX neurons co-release glutamate, in addition to other peptides such as DYN (Chou et al. 2001; Rosin et al. 2003; Schone et al. 2012; Muschamp et al. 2014). Consequently, the effects of ORX neuron activation on downstream targets are complex and may be multimodal depending on the nature of neurotransmitter/peptide cocktail released (Schone et al. 2014; Schone and Burdakov 2017). Exactly what might control this complex signaling is poorly understood, and may derive from activation of differential inputs. In addition, at least two subtypes of ORX neurons have been described based on physiological responses to glucose (Williams et al. 2008; Schone et al. 2011), which may contribute to heterogeneous output. The overall influence of ORX neuron activation and, in particular, ORX signaling through OX1R and OX2R is excitatory. However, multiple

factors including co-transmission of glutamate and inhibitory neuropeptides such as DYN result in complex signaling profiles, which likely contribute to dynamic effects on behavior. In addition, OX2R signaling can work through Gi/o pathways which can have an inhibitory effect on target neurons (Muroya et al. 2004). However, exactly how Gi/o interacts with Gq is unclear, and the Gi/o effects on alcohol use and other motivated behaviors are largely unknown.

3.3 ORX System and Motivational Behaviors

The ORX system has been implicated in a wide range of different behavioral functions (Willie et al. 2001; Sutcliffe and de Lecea 2002; Kuwaki and Zhang 2012; Giardino and de Lecea 2014; Mahler et al. 2014; Sakurai 2014; Flores et al. 2015; James et al. 2017a; Schone and Burdakov 2017). Early work focused on the role of ORX activity in the regulation of feeding and control of sleep and arousal. The association of the ORX system with feeding was initially based on the observation that intracerebroventricular ORX administration increased food intake (Sakurai et al. 1998). The relationship with sleep and arousal was initially based on the findings that the absence of ORX neurons and ORX in the CSF is a major, if not primary, factor in the disrupted sleep-wake balance seen in narcolepsy (Lin et al. 1999; Nishino et al. 2000; Thannickal et al. 2000). Many independent lines of research have validated both of these associations. Of particular interest with respect to the role of ORX in regulating motivation, ORX signaling is especially engaged when behavior is directed at highly palatable (rewarding) food such as chocolate, as opposed to rodent chow (Clegg et al. 2002; Cason et al. 2010). Thus, palatable foods that are sweet and high in fat drive activation of ORX neurons, and seeking of these rewarding substances are blocked with treatment with the OX1R antagonist SB334867 (Nair et al. 2008; Borgland et al. 2009; Choi et al. 2010). This finding that the ORX system is closely associated with highly reinforcing food rewards is relevant in the context of drugs of abuse and particularly alcohol. For both alcohol and other drugs of abuse, a number of studies have demonstrated parallel findings – that the ORX system plays an important role when motivation for the drug outcome is high (Borgland et al. 2009; Moorman and Aston-Jones 2009; Espana et al. 2010; Hollander et al. 2012; Mahler et al. 2014; Bentzley and Aston-Jones 2015; Lopez et al. 2016).

The ORX system also plays a critical role in regulating emotional state. In particular, this system has been strongly connected with regulation of stress, anxiety, and fear (Johnson et al. 2012; Kuwaki and Zhang 2012; Giardino and de Lecea 2014; Flores et al. 2015; James et al. 2017a), in part due to its influence on some of the systems that also control arousal (e.g., locus coeruleus) and motivation (e.g., BNST and amygdala). ORX neurons are activated following acute stress, and pharmacological or genetic decreases in ORX signaling result in blunted responses to stress challenges. ORX neurons also regulate fundamental physiological processes such as respiration, cardiovascular function, and temperature, via control of autonomic nuclei in the hypothalamus and brainstem (Madden et al. 2012; Kuwaki 2015; Carrive and Kuwaki 2017). Many of these functions are linked in order to regulate an overall adaptive active coping response to internal or external challenges.

3.4 Alcohol and the ORX System

A large body of evidence implicates a role for ORX signaling in alcohol- and drug-seeking/taking behaviors (Mahler et al. 2012; Baimel et al. 2015; Baimel and Borgland 2017; James et al. 2017b). In general, studies have shown that the ORX system is particularly involved in alcohol/drug-seeking behavior when motivation, demand, or effort requirements are high. This has led to the proposal that a major function of the ORX system is motivational activation, or to energize an individual to respond to needs, challenges, or potential rewards (Mahler et al. 2014). That this fundamental process gets coopted by alcohol and other drugs of abuse is important for understanding fundamental mechanisms of the addiction process. The relationship between ORX system function and motivational effects of alcohol was first investigated by Lawrence and colleagues in 2006 (Lawrence et al. 2006). Since then, there has been a growing interest in understanding contributions of the ORX system to alcohol use/misuse (Lawrence 2010; Mahler et al. 2012; Brown and Lawrence 2013; Barson and Leibowitz 2016; Walker and Lawrence 2017).

3.4.1 Effects of Alcohol Exposure on ORX Expression and Function in Brain

As noted above, the earliest demonstrations of a contribution of ORX signaling to alcohol seeking came from Lawrence and colleagues who reported increased prepro-ORX mRNA after chronic alcohol consumption (Lawrence et al. 2006). This was observed exclusively in alcohol-preferring iP rats originally derived from the Indiana University selectively bred line (Lumeng et al. 1977), but not in genetically selected non-preferring rats, supporting the notion that one link between ORX and alcohol is intensity of motivation or preference. More recent work by this group revealed no effect of alcohol self-administration on number of ORX positive neurons (Kastman et al. 2016), but a separate group demonstrated increased ORX mRNA following chronic alcohol, with expression levels correlated with preference (Barson et al. 2015). Other studies have reported mixed results as well. For example, both decreases and increases in ORX mRNA and peptide levels have been reported after acute or chronic alcohol administration in outbred rats (Morganstern et al. 2010). In studies involving binge-like and chronic alcohol drinking in mice, no changes in mRNA expression were noted, but decreases in ORX peptide levels were observed (Olney et al. 2015, 2017). Finally, zebrafish given chronic alcohol exposure exhibited signs of alcohol preference and increased ORX mRNA expression (Sterling et al. 2015), suggesting conservation of coarse aspects of encoding for this neuropeptide across species. Thus, while a number of studies have demonstrated that alcohol exposure influences ORX mRNA and peptide expression, differences in outcome likely reflect a number of differences in experimental parameters (e.g., species, alcohol dose, exposure duration).

A number of studies have demonstrated an association between alcohol exposure and activation of ORX neurons, primarily using the immediate early gene c-Fos as a measure of activation. For example, rats exhibiting relapse-like responding for alcoholic beer exhibited increased activation of ORX neurons, particularly in the lateral hypothalamus, which correlated significantly with intensity of responding

(Hamlin et al. 2007; Millan et al. 2010). Evidence for ORX neuron activation was also demonstrated in studies involving cue and context-related alcohol-seeking behavior in rats. In these studies, Fos activation of ORX neurons in the lateral hypothalamus was correlated with home-cage alcohol-seeking responses whereas context-driven reinstatement responding was correlated with ORX neuron activation in the dorsomedial and perifornical hypothalamic nuclei (Moorman et al. 2016). Discriminative-stimulus driven reinstatement in Wistar rats produced significant increases in c-Fos activation of ORX neurons across the lateral hypothalamus (Dayas et al. 2008), as did stress-induced reinstatement of alcohol responding in iP rats (Kastman et al. 2016) and sensitization following repeated alcohol injections in mice (Macedo et al. 2013). As with ORX mRNA expression, immunohistochemical measurements of ORX neuron activity present somewhat variable results across studies, particularly with respect to the relationship between specific behaviors and hypothalamic subregions, but they reliably show an impact of alcohol responding and consumption.

A small number of studies have investigated the relationship between ORX expression and alcohol use/dependence. Alcohol-dependent patients have been shown to exhibit higher levels of ORX expression during early (1–7 days) vs. late (multiple weeks) abstinence (Bayerlein et al. 2011). Similarly, plasma ORX levels were correlated with depression-like symptoms in early withdrawal in alcohol-dependent patients, but these correlations diminished after several weeks of continued abstinence (von der Goltz et al. 2011). Thus, although limited in scope, there is some clinical evidence to indicate a correlative relationship between ORX system activity and chronic alcohol use and withdrawal. The exact factors underlying these correlations (e.g., craving, stress of withdrawal, etc.) remain to be elucidated, but these studies point to the ORX system as a potential target for dependence treatment.

3.4.2 Effects of ORX Receptor Activation and Blockade on Alcohol-Related Behaviors

The majority of studies investigating the influence of the ORX system on alcohol-related behaviors have involved pharmacologically manipulating OXRs in mice and rats. In general, these studies have shown that antagonism of OX1Rs and, in some cases, OX2Rs results in reduced alcohol self-administration and relapse-like behavior. In many cases, the effect of OXR antagonism was found to be most robust in animals exhibiting high motivation for alcohol, suggesting a role for the ORX system in heightened levels of seeking and drinking as typically seen in alcohol dependence.

Antagonism of OX1Rs, particularly through systemic administration of drugs such as SB334867 (SB), has been shown to decrease motivational aspects of alcohol self-administration. For example, SB administration significantly decreased both operant responding for 10% alcohol and cue-induced reinstatement of alcohol seeking (Lawrence et al. 2006), findings that have been replicated in various rat strains at varying alcohol concentrations (Richards et al. 2008; Jupp et al. 2011b; Martin-Fardon and Weiss 2014; Moorman et al. 2017). Systemic SB treatment also decreased stress (yohimbine)-induced alcohol relapse-like behavior (Richards et al. 2008) and

discriminative stimulus-induced reinstatement responding (Jupp et al. 2011a). Neuropeptide S infused into the lateral hypothalamus also induced reinstatement of alcohol seeking, and this behavior was significantly reduced by pretreatment with SB (Cannella et al. 2009). Neuropeptide S may have direct effects on ORX neurons via a Gq/s protein-coupled receptor (NPSR), as over 40% of ORX neurons exhibit NPSR expression and neuropeptide S axons are found in apposition to ORX neurons (Ubaldi et al. 2016). OX1R antagonism decreased progressive ratio breakpoint for alcohol, but not sucrose, suggesting a potentially unique role in alcohol vs. natural reward motivation (Jupp et al. 2011b). SB treatment also decreased alcohol consumption when it was offered in the home-cage, as did the dual OX1R/OX2R antagonist almorexant. In contrast, selective antagonism of OX2Rs (with LSN2424100) had no effect on alcohol drinking (Anderson et al. 2014; Moorman and Aston-Jones 2009). Another study corroborated these findings, demonstrating that systemic almorexant treatment decreased operant alcohol self-administration, although sucrose self-administration was also influenced (Srinivasan et al. 2012). Decreased alcohol consumption following OX1R antagonism has also been shown in mouse models of heavy drinking, such as binge-like consumption and escalated alcohol intake resulting from repeated cycles of chronic intermittent ethanol (CIE) vapor exposure (Carvajal et al. 2015; Olney et al. 2015; Lopez et al. 2016). In a model of compulsive-like alcohol drinking (C57BL/6J mice exhibiting aversion resistance to quinine-adulterated alcohol), OX1R antagonism (SB), but not OX2R antagonism (TSC-OX2-29) reduced intake of alcohol presented alone or in combination with quinine (Lei et al. 2016a). The OX1R antagonist SB pretreatment also blocked alcohol conditioned place preference and alcohol sensitized hyperlocomotion in mice (Voorhees and Cunningham 2011; Macedo et al. 2013). Taken together, a substantial body of evidence has emerged indicating that OXR antagonism, in particular OX1R antagonism, decreases motivational aspects of alcohol self-administration behavior.

One observation that appears consistent across a number of studies is the finding that OXR antagonism is more potent and/or efficacious when motivation for alcohol seeking and consumption is at a high level, either due to natural variation in alcohol preference, or through measures employed to produce dependence-like states. Support for this contention comes from studies demonstrating that the OX1R antagonist SB produces more robust decreases in alcohol self-administration and relapse-like behavior in rats genetically selected for high alcohol preference (Lawrence et al. 2006; Dhaher et al. 2010; Anderson et al. 2014), as well as outbred rats with a high propensity for alcohol taking behavior (Moorman and Aston-Jones 2009; Moorman et al. 2017). Further, OX1R antagonism, using either SB or another selective antagonist (GSK1059865), selectively decreased escalated drinking in dependent (CIE-exposed) mice without altering more moderate levels of alcohol intake in nondependent mice (Lopez et al. 2016). Finally, OX1R antagonism was found to be more effective in reducing compulsive-like alcohol drinking in quinine-resistant (but not quinine-sensitive) mice (Lei et al. 2016a). Collectively, these findings may have important clinical implications, as the ORX system may be a particularly attractive target for treatment of individuals that have transitioned to heavy, compulsive-like alcohol drinking.

Despite this growing and compelling evidence, there are some instances in which either OX1R antagonism has had limited effects or OX2R antagonism has been shown to more prominently influence alcohol self-administration behavior. For example, in one study SB treatment had no effect on Pavlovian spontaneous recovery of alcohol seeking following a period of extinction training, but the drug did decrease renewal of rewarded alcohol self-administration in female alcohol-preferring rats (Dhaher et al. 2010). This raises questions about potential sex differences in a line of research primarily dominated by studies of male rodents. Other studies have found no effect of OX1R antagonism (using the SB-408124 compound) on alcohol self-administration or conditioned place preference but, instead, have observed an influence of OX2R antagonism, using the JNJ-10397049 compound (Shoblock et al. 2011). In contrast to some studies described above, progressive ratio breakpoints in alcohol preferring P rats were not affected by SB treatment (nor by OX2R antagonism), but were decreased by the dual OX1R/OX2R antagonist almorexant (Anderson et al. 2014). Mice in this study exhibited decreased alcohol and sucrose drinking with all OXR antagonists, suggesting a potent effect of OXR manipulation on reward consumption in general. In a separate study in mice, alcohol conditioned place preference was only modestly influenced by SB treatment, although alcohol-induced hyperlocomotion was decreased (Voorhees and Cunningham 2011). Knockdown of ORX expression using morpholinos had limited effect on responding for alcoholic beer, raising questions about the exact nature of ORX control over alcohol-seeking behavior (Prasad and McNally 2014). Thus, although the majority of pharmacological findings relating ORX to motivational effects of alcohol implicate a role for OX1Rs, there are certainly exceptions to this rule. These divergent findings suggest a complex mechanism by which the ORX system regulates alcohol seeking and consumption, potentially by signaling at different receptors in different brain areas.

3.5 Brain Circuitry Analyses of ORX System Involvement in Alcohol

As noted above, the ORX system projects widely across the brain. Systemic treatment with OXR antagonists has been shown to exert direct and indirect effects on behaviors related to the rewarding effects of alcohol. To further investigate these effects, recent studies have begun probing brain region-specific OXR signaling in the context of alcohol-related behaviors. Results from these studies demonstrate a complex framework in which OX1R signaling in some brain areas regulates alcohol self-administration behavior whereas OX2R signaling influences it in other brain regions.

Perhaps the most salient target region for ORX signaling is the VTA, given its prominent role in reward and alcohol/drug-motivated behaviors (Aston-Jones et al. 2010; Brown and Lawrence 2013; Baimel and Borgland 2017). The OX1R antagonist SB applied directly into the VTA decreased cue-induced reinstatement of alcohol responding in iP preferring rats (Brown et al. 2016). The dual OX1R/OX2R antagonist almorexant injected into the VTA decreased self-administration of both alcohol (20%) and sucrose (5%) in Long-Evans rats (Srinivasan et al. 2012; Prasad and McNally 2014). In contrast to these results, SB application to the VTA did not reduce Neuropeptide

S-enhanced reinstatement of alcohol seeking in rats, nor did administration of the drug into the noradrenergic locus coeruleus (Ubaldi et al. 2016). Further, SB (and not the OX2R antagonist TCS-OX2-29) injected into the VTA decreased alcohol consumption during the first hour of consumption in a mouse model of binge-like drinking (Olney et al. 2017). Taken together, there is some evidence indicating that ORX signaling in the VTA may contribute to regulation of alcohol self-administration and relapse-like behavior.

Other brain regions where ORX signaling could potentially influence alcohol-seeking/drinking behaviors include the PFC and NAc (Kalivas et al. 2005; Kalivas 2008; Barker et al. 2015; Marchant et al. 2015). Systemic SB treatment that decreased reinstatement of alcohol seeking also decreased c-Fos expression in the NAc core, medial prefrontal cortex (mPFC), orbitofrontal cortex, and piriform cortex (Jupp et al. 2011a). Targeted SB application into the mPFC in iP rats significantly decreased cue-induced reinstatement of alcohol (but not sucrose) responding (Brown et al. 2016). SB injected into the shell subdivision of the NAc or the mPFC decreased alcohol intake in mice (Lei et al. 2016b), and direct administration of SB into the NAc shell region decreased alcohol self-administration in mice (Lei et al. 2016b) and rats (Mayannavar et al. 2016). In contrast, no effect of SB treatment to the insula was observed (Lei et al. 2016b), which is interesting given the important role for the insula in regulating alcohol-seeking behavior (Seif et al. 2013) and the noted influence of ORX in the insula on nicotine seeking (Hollander et al. 2008). In total, however, these findings demonstrate a role for ORX signaling in both cortical (mPFC) and striatal (NAc) regions in regulation of alcohol seeking and consumption.

A number of other brain regions have been implicated in mediating effects of ORX system activity on alcohol-related behaviors. Injections of ORX-A in both the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus increased alcohol consumption (Schneider et al. 2007), potentially through increasing the frequency of drinking bouts (Chen et al. 2014). SB infusion into the PVN blocked the effects of intra-lateral hypothalamus injection of Neuropeptide S on reinstatement of alcohol responding, as did SB infusion into the BNST (Ubaldi et al. 2016). Antagonism of both OX1Rs and OX2Rs in the CeA reduced alcohol intake in a mouse binge-drinking model (Olney et al. 2017). ORX also interacts with relaxin-3/RXFP3, another peptide system implicated in alcohol-seeking behavior (Ryan et al. 2014). ORX-A, signaling through OX2Rs, excites relaxin-3 neurons in the nucleus incertus, and OX2R antagonists (but not OX1R antagonists) infused into the nucleus incertus decrease stress-induced reinstatement of alcohol responding in alcohol preferring iP rats (Kastman et al. 2016). Another nucleus that may regulate alcohol seeking through OXR2 signaling is the paraventricular nucleus of the thalamus (PVT). This area receives strong ORX projections and is gaining attention as a potential major regulator of motivated behavior and drug seeking (Martin-Fardon and Boutrel 2012; James and Dayas 2013; Matzeu et al. 2014, 2016), including alcohol-seeking behavior (Hamlin et al. 2009). Recent work has shown that alcohol drinking increases ORX peptide and OX2R expression in the anterior PVT, that ORX-A and ORX-B infusions into the anterior PVT increase alcohol intake, and that OXR antagonists, particularly those targeting OX2Rs, in the anterior PVT decrease alcohol consumption (Barson et al. 2015), potentially through

interaction with the substance P system (Barson et al. 2017). Thus, ORX system activity within a number of brain regions appears to play a role in modulating alcohol-related behaviors. Future work in this area will be critical for understanding the anatomical and network basis for these effects.

4 Potential Overlap Between ORX and DYN/KOR Systems in Mediating Alcohol-Related Behaviors

At first glance it may seem counterintuitive to group the ORX and DYN systems when considering peptidergic regulation of alcohol seeking, particularly when considering the diversity of neuropeptides that influence alcohol actions (Barson and Leibowitz 2016). However, there are a number of interesting intersection points to consider in this regard. Most prominently, almost all ORX neurons co-express DYN, and both peptides are packaged in the same vesicles and are co-released (Chou et al. 2001; Crocker et al. 2005; Li and van den Pol 2006; Muschamp et al. 2014; Baimel and Borgland 2017), although ORX is not found in DYN neuron populations outside the lateral hypothalamus. These findings indicate a close degree of coupling between ORX and at least one population of DYN neurons. That is, when "ORX" neurons in the lateral hypothalamus are activated, so are "DYN" neurons. Furthermore, OXRs and KORs are located in many of the same regions, including those in which both peptides are known to regulate motivational effects of alcohol (e.g., VTA, NAc, BNST, CeA, and PFC) (Fig. 1). Although DYN projections to each of these regions

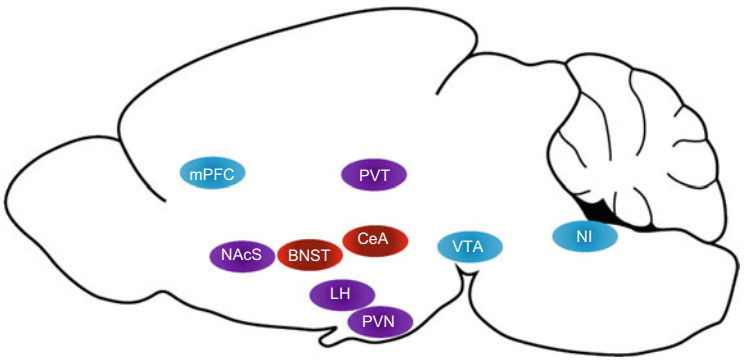

Fig. 1 Brain regions in which pharmacological manipulation of kappa opioid receptors (red), orexin receptors (blue), or both (purple) influences ethanol consumption are shown. Blockade of orexin receptors in the medial prefrontal cortex (mPFC), ventral tegmental area (VTA), nucleus incertus (NI), and paraventricular nucleus of the thalamus (PVT) results in decreased drinking. Conversely, activation of orexin receptors in the PVT increases drinking. Orexin and kappa opioid receptor agonists exert opposing effects on ethanol intake in the lateral hypothalamus (LH) and paraventricular nucleus of the hypothalamus (PVN), with orexin agonists increasing drinking and kappa opioid receptor agonists decreasing drinking. Antagonism of both orexin and kappa opioid receptors in the nucleus accumbens shell (NAcS) reduce ethanol consumption. Within the central nucleus of the amygdala (CeA), blockade of kappa opioid receptors reduces ethanol intake. Mouse brain outline by Jonas Töle

may originate from multiple sources, the shared receptor profiles across the two systems suggest a possible interaction in signaling.

ORX and DYN have largely opposing physiological effects, with OXR signaling primarily producing excitatory effects and KOR signaling producing inhibitory responses in postsynaptic neurons. This raises an interesting question about what purpose is served by co-release of these peptides. Individual dopamine neurons of the VTA are most commonly responsive to both peptides, though some neurons are selectively responsive to ORX vs. DYN (Muschamp et al. 2014). Similar effects have been seen in the basal forebrain (Ferrari et al. 2016), and will presumably be discovered in other co-target regions. The interplay between these systems may also serve as feedback or gating mechanisms within the lateral hypothalamus, as ORX activates ORX/DYN neurons via glutamatergic interneurons (Li et al. 2002), and these neurons are directly inhibited by DYN (Li and van den Pol 2006). These initial studies suggest that either a balance of ORX/DYN release or a balance of OXR/KOR expression and function regulates excitatory/inhibitory profiles. Alternately, it is possible that co-release is precisely balanced to produce a hybrid response that is neither purely excitatory nor inhibitory, but perhaps involves more fine-tuned responses mediated by specific intracellular signaling pathways (Robinson and McDonald 2015). The interaction may also involve differential regulation of inputs or outputs depending on which other pathways are engaged during behavior (Baimel et al. 2017). It is also of note that expression of these peptides is under control of different promoters, which may be activated at different times during behavior. So, synthesis and release may be differentially regulated by the ORX/DYN neurons themselves.

The ORX and DYN systems also mediate different behavioral profiles – ORX more appetitive, DYN more aversive. ORX may facilitate reward motivation by occluding DYN anti-reward signaling, as recently demonstrated in the first behavioral study to directly address this question (Muschamp et al. 2014). Systemic or intra-VTA SB increased brain stimulation threshold levels and decreased impulsivity, and either SB treatment in rats or OXR1 knockdown in mice reduced cocaine self-administration. Interestingly, pretreatment with the KOR antagonist nor-BNI ameliorated all of these changes in behavior. This provides support for the fact that, at least in the VTA and probably elsewhere, the ORX and DYN systems serve opposing or regulatory roles over one another. On the other hand, binary distinctions between the two systems may be unrealistic, given that ORX signaling is also associated with stress and arousal. Despite knowing for over 15 years that these systems overlap, we are still at the beginning of understanding the meaning of ORX/DYN co-expression and co-release. Exactly how these interactions contribute to motivational effects of alcohol, and the therapeutic potential of these interactions, remains to be investigated.

Despite some physiological and behavioral differences, there are significant commonalities between ORX and DYN systems that are particularly relevant with regard to their influence on the motivational effects of alcohol. Orexins are known to play a role in the regulation of food/water intake (e.g., Sakurai et al. 1998; Kunii et al. 1999) and dynorphins have been shown to influence consumption of food and palatable solutions (e.g., Morley and Levine 1983; Beczkowska et al. 1992). These

peptides may exert their effects to a greater degree under conditions of high motivation (hunger or thirst resulting from deprivation). Likewise, both systems have been shown to be especially effective in altering alcohol drinking when motivation for the drug is high; the effects of OXR and KOR manipulation on alcohol consumption are greater in subjects that exhibit higher levels of drinking and in models of binge-drinking and dependence-related escalated drinking (Lindholm et al. 2001; Lawrence et al. 2006; Walker and Koob 2008; Moorman and Aston-Jones 2009; Anderson et al. 2014, 2016; Olney et al. 2015; Lopez et al. 2016). Interestingly, under these conditions of apparent high motivation for alcohol, pharmacological manipulation of ORX and KOR receptor systems is selective in influencing alcohol intake relative to motivation for natural rewards (e.g., sucrose). Further, ORX and DYN may play an enhanced role in signaling stress and arousal in these circumstances, as these behavioral components are integral features of motivated action that help focus attention on the target goal. Thus, motivation for high levels of alcohol consumption may result, at least in part, from activation of these peptide systems in a manner that redirects their role from regulating motivation towards natural rewards to driving elevated motivational states that engender high alcohol intake.

Although the exact mechanisms by which ORX and DYN systems influence alcohol seeking and consumption are not fully understood, it is reasonable to assume that chronic alcohol-induced adaptations in these systems contribute to the more selective effects on self-administration associated with dependence. Synaptic plasticity of DYN and ORX neurons induced by chronic alcohol, as observed in studies of other drugs of abuse, is a likely mechanism (Li and van den Pol 2008; Sirohi et al. 2012; Yeoh et al. 2012; Rao et al. 2013). However, the precise adaptations that may occur upstream to alter excitability in DYN and ORX neurons remain unknown. One possibility may involve selective alteration of different pathways for these peptides following chronic alcohol exposure. That is, chronic alcohol may weaken the synaptic strength of certain inputs associated with pathways that subserve motivational behavior directed at natural rewards while simultaneously enhancing synapses from regions that are especially activated by alcohol-associated cues or stress. Alternately, chronic alcohol may produce synaptic changes in downstream targets to promote enhanced motivation for alcohol, as has been seen in studies of psychostimulants and opioids (Baimel et al. 2015), either through direct changes in peptidergic signaling at targets or indirectly through enhancement or suppression of glutamatergic or GABAergic signaling, as observed in ORX and DYN signaling in the VTA (Margolis et al. 2005; Borgland et al. 2006, 2008). Yet another possibility is that chronic alcohol exposure may produce changes at a genomic level within these neural populations. That is, promoters may be up- or down-regulated, biasing regulation and activity of specific DYN and ORX pathways that underlie motivational behavior. Supporting this idea, alcohol exposure in the dorsal striatum was shown to activate brain-derived neurotrophic factor (BDNF) signaling cascades that result in elevated preprodynorphin mRNA and increased DYN translation; in turn, DYN signaling was shown to mediate the decreased alcohol consumption associated with increased BDNF (Logrip et al. 2008).

These considerations are relatively speculative, largely because there is a general paucity of information regarding mechanisms by which chronic alcohol exposure

functionally alters DYN and ORX systems. Future work utilizing contemporary experimental approaches will no doubt further advance our understanding of how chronic alcohol influences these peptide systems at the molecular, neuronal, and circuitry levels of analyses. This work, in turn, will shed valuable insight regarding the viability of targets within the DYN and ORX systems as potential therapeutics for tempering excessive alcohol consumption.

5 Summary

A large body of evidence indicates that both DYN and ORX are associated with stress and reward motivation, with implications that these neuropeptide systems play a significant role in contributing to psychiatric disorders including anxiety, depression, and addiction. The neuroanatomical distribution of both neuropeptide systems overlaps in brain regions implicated in the motivational effects of alcohol, including the PFC, NAc, BNST, CeA, and VTA. Accordingly, numerous reports have indicated that both DYN and ORX modulate alcohol intake, particularly when motivation to consume alcohol is high, suggesting that both neuropeptide systems may be promising therapeutic targets for the treatment of alcohol dependence. Interestingly, despite evidence that ORX is typically colocalized with DYN, and that co-release of these neuropeptides can produce opposing effects on dopamine neurons in the VTA, the implications of these interactions have not been studied within the context of alcohol reward. Future work disentangling selective vs. interactive contributions of these neuropeptide systems holds great promise for development of new and novel treatment approaches for alcohol use disorders.

Acknowledgments This work was supported by NIH grants F32 AA023700 (RIA), P50 AA010761 (HCB), U01 AA014095 (HCB), U01 AA020929 (HCB), AA024571 (DEM), AA025481 (DEM), DA041674 (DEM), VA Medical Research BLR&D BX000813 (HCB), and a NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation (DEM).

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Transcriptional Regulators as Targets for Alcohol Pharmacotherapies

Antonia M. Savarese and Amy W. Lasek

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Abstract

Alcohol use disorder (AUD) is a chronic relapsing brain disease that currently afflicts over 15 million adults in the United States. Despite its prevalence, there are only three FDA-approved medications for AUD treatment, all of which show limited efficacy. Because of their ability to alter expression of a large number of genes, often with great cell-type and brain-region specificity, transcription factors

The original version of this chapter was revised. A correction to this chapter is available at https://doi.org/10.1007/164_2018_194.

A. M. Savarese
Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, USA

Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR, USA
e-mail: savarese@ohsu.edu

A. W. Lasek (✉)
Department of Psychiatry, University of Illinois at Chicago, Chicago, IL, USA
e-mail: alasek@uic.edu

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_101

and epigenetic modifiers serve as promising new targets for the development of AUD treatments aimed at the neural circuitry that underlies chronic alcohol abuse. In this chapter, we will discuss transcriptional regulators that can be targeted pharmacologically and have shown some efficacy in attenuating alcohol consumption when targeted. Specifically, the transcription factors cyclic AMP-responsive element binding protein (CREB), peroxisome proliferator-activated receptors (PPARs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and glucocorticoid receptor (GR), as well as the epigenetic enzymes, the DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), will be discussed.

Keywords
CREB · DNA methylation · DNA methyltransferase · DNMT · Glucocorticoid receptor · HDAC · Histone acetylation · Histone deacetylase · Nuclear factor kappa B · PPAR

1 Introduction

Alcohol (ethanol) induces both rapid changes in receptor signaling and the longer-acting second messenger signal transduction cascades in the brain that culminate in chromatin remodeling and changes in gene expression. While acute alcohol can lead to transient changes in these signaling pathways, chronic alcohol use leads to persistent genome-wide epigenetic modifications and associated changes in gene expression that alter the neuronal circuitry involved in alcohol reward, craving, and the negative affective state that develops during ethanol withdrawal. Transcription factors and epigenetic modifiers therefore represent excellent targets for attenuating or reversing the pathological effects of chronic alcohol use on neuronal circuitry and ameliorating alcohol use disorder (AUD). In this chapter, we will discuss the role of transcription factors and chromatin-modifying enzymes in alcohol consumption and behaviors related to problematic alcohol use. Many pharmacological agents targeting transcriptional regulators and epigenetic enzymes have been developed that have shown efficacy in preclinical models of AUD.

2 Transcription Factors

2.1 Cyclic AMP-Responsive Element Binding Protein

Cyclic AMP-responsive element binding protein (CREB) is a transcription factor that is widely expressed in the nervous system and is critically involved in neuronal development, plasticity, and learning and memory (Silva et al. 1998). Activity of CREB is modulated by phosphorylation by a number of kinases and phosphatases, including protein kinase A (PKA, Fig. 1a) and calcium/calmodulin-dependent protein kinases (Soderling 1999; Mayr and Montminy 2001). Phosphorylated CREB (pCREB) binds to its coactivator CREB binding protein (CBP), a histone acetyltransferase (HAT) that

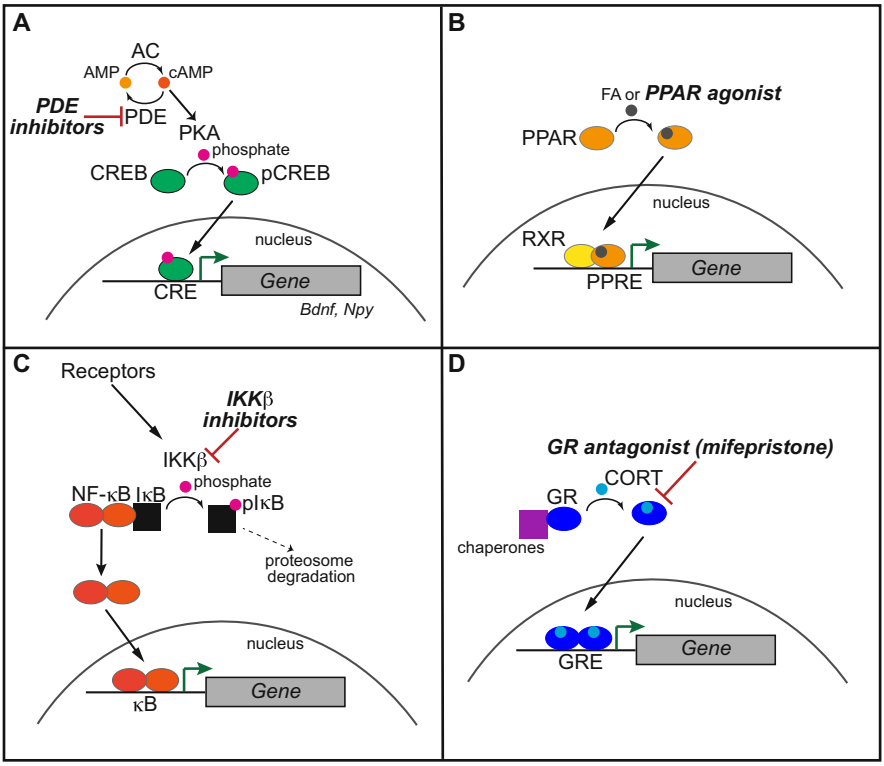

Fig. 1 Simplified diagram of transcriptional pathways and targets for intervention for alcohol use disorder (AUD) treatment. (**a**) The cAMP-responsive element binding protein (CREB) pathway. Adenylyl cyclase (AC) produces cAMP from AMP, activating protein kinase A (PKA). CREB is phosphorylated (pCREB) by several kinases, one of which is PKA. Once phosphorylated, CREB translocates to the nucleus and binds to cAMP-responsive elements (CRE) in the DNA to activate transcription of genes associated with AUD such as *Bdnf* and *Npy*. One method to activate CREB is to use compounds that inhibit the phosphodiesterases (PDEs) that hydrolyze cAMP, thus increasing cAMP levels and activating PKA. PDE inhibitors reduce alcohol consumption in animal models of AUD. (**b**) The peroxisome proliferator-activated receptor (PPAR) signaling pathway. PPARs are activated by their endogenous ligands, fatty acids (FA), or by synthetic agonists such as the thiazolidinediones and fibrates. Upon ligand binding, PPARs translocate to the nucleus and interact with retinoid X receptor (RXR) at peroxisome proliferator response elements (PPREs) to regulate gene transcription. PPAR agonists reduce alcohol consumption in animal models of AUD. (**c**) The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. NF-κB exists as a dimer of different subunits and is complexed with an inhibitory molecule, inhibitor κB (IκB) in the cytosol. Activation of various receptors leads to activation of IκB kinase (IKKβ) and phosphorylation of IκB. This event targets IκB for degradation, releasing NF-κB for translocation to the nucleus to regulate gene expression at κB elements. IKKβ inhibitors reduce alcohol consumption in mice. (**d**) Glucocorticoid receptor (GR) pathway. GR is held in the cytosol by chaperone proteins. Once bound to its ligand, cortisol (in humans/nonhuman primates) or corticosterone (in rodents) (CORT), GR translocates to the nucleus and binds to glucocorticoid response elements (GREs) to regulate gene transcription. The GR antagonist mifepristone has shown efficacy in reducing alcohol consumption in rodents and humans

acts to open chromatin and activate transcription (see Sect. 3), and this complex then binds to cAMP-response elements (CREs) in the DNA. As such, CREB activity is tightly regulated and can rapidly change to adapt to different stimuli.

Polymorphisms in both the CREB1 gene (rs35349697) and the CBP gene CREBBP (rs3025684) are associated with alcohol addiction (Pal et al. 2014; Kumar et al. 2011). Two key transcriptional target genes of CREB are brain derived neurotrophic factor (*BDNF*) and neuropeptide Y (*NPY*) (Tao et al. 1998; Pandey et al. 2004). Polymorphisms in *NPY* have been associated with alcohol consumption, and a large body of literature has demonstrated that manipulation of the NPY system in rodents alters ethanol consumption (reviewed in Robinson and Thiele 2017). BDNF modulates neuronal development, differentiation, and survival and has been implicated in most psychiatric disorders, including addiction (Moonat et al. 2011; Greenwald et al. 2013; Lobo et al. 2010; Logrip et al. 2015). A single nucleotide polymorphism (SNP) of *BDNF* (Val66Met) has been associated with alcohol dependence. Minor allele carriers exhibit resistance to relapse (Wojnar et al. 2009) and decreased brain activation in networks associated with more severe dependence symptoms (Chen et al. 2015). Admittedly, this association has not always been found (Nedic et al. 2013; Forero et al. 2015), and discrepancies in study results may be partly attributable to differences in the frequency of the *BDNF* Val66Met allele across ethnic populations (Shimizu et al. 2004; Pivac et al. 2009).

Alcohol regulates CREB activity by modulating its phosphorylation. Acute ethanol treatment increases, while chronic ethanol attenuates, pCREB (Yang et al. 1998; Pandey et al. 2004). Similarly, withdrawal from ethanol after chronic exposure is characterized by decreased pCREB, without changes in total CREB (Pandey et al. 2001). In vitro, acute ethanol induces an increase in gene expression that is dependent on CREB phosphorylation, an effect that can be blocked by inhibiting PKA activity (Asher et al. 2002). It is possible that the decreased pCREB that results from chronic alcohol exposure is a direct result of reduced PKA activity. Chronic intermittent alcohol exposure in rats results in increased expression of protein kinase A inhibitor alpha (-PKI-alpha), a member of a family of proteins implicated in reducing PKA activity (Repunte-Canonigo et al. 2007).

Changes in pCREB may mediate behaviors at each phase of the alcohol addiction cycle. The development of tolerance to the sedative effect of alcohol is associated with increased pCREB (Yang et al. 2003). Additionally, rats selectively bred for high alcohol consumption (alcohol-preferring, or P rats) have decreased CREB expression, pCREB, and CRE-DNA binding in the amygdala compared to their alcohol non-preferring counterparts (Pandey et al. 1999). Withdrawal from chronic alcohol exposure leads to decreased pCREB in the amygdala (Pandey et al. 2001). This reduction of pCREB in the amygdala, a brain region critical for anxiety-like behavior, is associated with both high anxiety and increased ethanol preference (Pandey et al. 2003). Similarly, mice that are deficient in CREB display more anxiety-like behavior relative to wild-type mice and show an attenuation of ethanol-induced anxiolysis (Pandey et al. 2004). Importantly, restoring CREB function in the amygdala of P rats can reduce both alcohol intake and anxiety-like behavior (Pandey et al. 2005).

Although there are no pharmacological agents that directly interact with CREB, CREB activity can be increased indirectly by elevating cAMP levels and activating

Table 1 Compounds that act on phosphodiesterases (PDEs) and decrease ethanol consumption

Compound	Target	Species tested	Approved for clinical use	References
Rolipram	PDE4	Mice, rats	No	Hu et al. (2011), Blednov et al. (2014), Liu et al. (2017) Wen et al. (2012) and Franklin et al. (2015)
Roflumilast	PDE4	Mice	Yes[a]	Liu et al. (2017)
Ibudilast	PDE (nonselective)	Rats, mice	Yes[b]	Bell et al. (2015)
Ro 20-1724	PDE4	Mice, rats	No	Hu et al. (2011) Franklin et al. (2015)
TP-10	PDE10A	Rats	No	Logrip et al. (2014)
Mesopram	PDE4	Mice	No	Blednov et al. (2014)
CDP 840	PDE4	Mice	No	Blednov et al. (2014)
Piclamilast	PDE4	Mice	No	Blednov et al. (2014)

[a]Approved for the treatment of chronic obstructive pulmonary disease (COPD)
[b]Approved in Japan for the treatment of asthma, multiple sclerosis, and cerebrovascular disorders

PKA, which is achieved by inhibition of the phosphodiesterases (PDEs) that hydrolyze cAMP (Fig. 1a). Two recent review articles have discussed the effects of PDE inhibitors on alcohol consumption (Logrip 2015; Olsen and Liu 2016), so the results will only be briefly summarized here (Table 1). Rolipram, a phosphodiesterase-4 (PDE4) inhibitor, increases pCREB in the brain (Hu et al. 2016) and reduces alcohol intake in rats (Franklin et al. 2015; Wen et al. 2012) and in mice (Hu et al. 2011; Blednov et al. 2014; Liu et al. 2017). Other PDE4 inhibitors that reduce ethanol consumption in rodents are roflumilast, Ro 20-1724, mesopram, CDP 840, and piclamilast (Table 1). In addition, the PDE10 inhibitor TP-10 reduces ethanol self-administration by rats, and the nonselective PDE inhibitor ibudilast reduces ethanol consumption by high-drinking rats and ethanol-dependent mice (Bell et al. 2015; Logrip 2015). Most of the aforementioned studies observed a selective reduction in ethanol intake with no change in water or saccharin intake, but others have shown reductions in saccharin or sucrose intake with administration of TP-10 and rolipram (Logrip 2015; Franklin et al. 2015). This initial anhedonic behavior is likely a short-term side effect of drug administration. Rolipram, for instance, initially reduced sucrose intake in P rats, but intake normalized after 5 days of exposure, while suppression of ethanol intake continued (Franklin et al. 2015).

Ibudilast was recently tested in human subjects with mild to severe AUD and was found to improve mood after exposure to an alcohol cue or stress, and reduced craving, but did not change the subjective effects of alcohol (Ray et al. 2017). Ibudilast is currently approved in Japan for the treatment of asthma, multiple sclerosis, and cerebrovascular disease and is generally considered safe. However, ibudilast has gastrointestinal side effects that include nausea, vomiting, and abdominal pain. Roflumilast is a selective PDE4 inhibitor that is FDA-approved for the treatment of chronic obstructive pulmonary disease (COPD) and also has gastrointestinal side effects. Another PDE inhibitor that is currently being used clinically for the treatment of psoriasis is apremilast, a selective PDE4 inhibitor that may have fewer side effects, but animal and

human studies need to be completed to evaluate whether it can reduce ethanol consumption. PDE inhibitors may, in fact, reduce drinking through their anti-inflammatory actions (Page and Spina 2011). PDE inhibitors reduce inflammatory neuroimmune responses, which are induced after chronic alcohol exposure and have emerged as important promoters of excessive alcohol intake (Crews et al. 2017; Titus et al. 2015). Thus, the ability of PDE inhibitors to reduce ethanol consumption may not necessarily be solely related to the enhancement of CREB signaling.

2.2 Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors belonging to the nuclear hormone receptor superfamily. PPARs are distributed throughout the body and contribute to a range of biological processes (for a review, see Berger and Moller 2002). Although primarily known for their role in regulating lipid metabolism, PPARs have also been shown to play a role in neuroprotection through repression of pro-inflammatory genes, including the inducible nitric oxide synthase gene (Pascual et al. 2005). PPARs function by translocating to the nucleus upon ligand binding (Fig. 1b). Saturated and unsaturated fatty acids, and their derivatives, are the endogenous ligands of PPARs, although a number of synthetic ligands have also been developed (Berger and Moller 2002). Once in the nucleus, PPARs form heterodimers with retinoid X receptors (RXRs) and bind to PPAR response elements (PPREs) in the promoter region of target genes. Coactivator proteins, such as steroid receptor coactivator-1 (SRC-1), then bind to the transcriptional complex to help initiate transcription (Zhu et al. 1996). The efficiency of coactivator proteins to aid in transcription depends upon which ligand is bound to the PPAR complex, allowing for dynamic control of PPAR target gene expression (Berger and Moller 2002).

Three isoforms of PPARs exist: PPARα, PPARγ, and PPARβ/δ. While most regions of the brain express all three isoforms, PPARβ/δ has the most widespread distribution and dense expression in the rat brain, with PPARγ showing the most restricted expression pattern (Moreno et al. 2004). All three isoforms may work in a coordinated fashion, with PPARβ/δ regulating the activity of the other two PPAR types (Aleshin et al. 2013). Importantly, PPARs are expressed in regions of the brain critical to addiction (i.e., the nucleus accumbens, ventral tegmental area, and amygdala) (Warden et al. 2016) and have recently been implicated in the addiction cycle (Flores-Bastias and Karahanian 2018). Data from a genome-wide association study (GWAS) of the genetics of alcoholism, the Collaborative Study on the Genetics of Alcoholism (COGA), supported an association with the genes encoding PPARγ and PPARα with alcohol withdrawal (Blednov et al. 2015). Intriguingly, while no genetic association was found in that GWAS study for PPARβ/δ, individuals with an AUD were shown to have altered expression of PPARβ/δ and PPARG coactivator 1 alpha (PGC-1alpha) protein in the amygdala and cortical regions of the brain (Ponomarev et al. 2012). Alterations in expression of PPARβ/δ in the brain may then be a consequence of chronic alcohol use. Alternatively, discrepancies in the results of

Table 2 Compounds that act on transcriptional regulators and decrease ethanol consumption

Compound	Target	Species tested	Approved for clinical use	References
Gemfibrozil	PPARα	Rats	Yes[a]	Barson et al. (2009)
Pioglitazone	PPARγ	Rats, mice	Yes[b]	Stopponi et al. (2011, 2013) and Blednov et al. (2015)
Rosiglitazone	PPARγ	Rats	Yes[b]	Stopponi et al. (2011)
Fenofibrate	PPARα	Mice, rats	Yes[a]	Blednov et al. (2015, 2016), Karahanian et al. (2014), and Ferguson et al. (2014)
Tesaglitazar	PPARα/γ	Mice	No	Blednov et al. (2015, 2016) and Ferguson et al. (2014)
Bezafibrate	PPARα/γ/δ	Mice	Yes[a]	Blednov et al. (2015)
TPCA-1 (2-[(aminocarbonyl) amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide)	IKKβ	Mice	No	Truitt et al. (2016)
Sulfasalazine	IKKβ	Mice	Yes[c]	Truitt et al. (2016)
Mifepristone	GR/progesterone receptor	Rats, humans	Yes[d]	Vendruscolo et al. (2012, 2015)
CORT113176	GR	Rats	No	Vendruscolo et al. (2015)

[a]Approved treatments for lowering high cholesterol and triglycerides
[b]Approved for the treatment of type 2 diabetes
[c]Approved for the treatment of Crohn's disease and rheumatoid arthritis
[d]Approved for use in terminating pregnancy and in controlling hyperglycemia in individuals with Cushing's syndrome

these two studies could suggest that changes in epigenetic regulation of PPARβ/δ may increase risk for the development of an AUD.

Several agonists of PPARs have proven efficacious in regulating alcohol intake in animal models (Table 2). Agonists of PPARγ and PPARα (pioglitazone, fenofibrate, and tesaglitazar), but not PPARβ/δ (GWO742), decreased alcohol intake and preference in C57BL/6J mice (Blednov et al. 2015; Ferguson et al. 2014). Furthermore, fenofibrate and tesaglitazar suppressed ethanol intake in wild-type mice but had no effect on PPARα null mice, supporting a direct role of PPARα in regulating drinking (Blednov et al. 2016). Interestingly, PPARα may be acting in a sex-dependent manner to regulate ethanol intake; while male mice showed reductions in ethanol intake with fenofibrate and tesaglitazar, female mice showed no response to fenofibrate and an attenuated response to tesaglitazar relative to male mice (Blednov et al. 2016).

Ferguson and colleagues collected amygdala and prefrontal cortex in mice that were given agonists to PPARα (fenofibrate), PPARα/γ (tesaglitazar), or PPARα/γ/β

(bezafibrate) and, by using gene expression microarrays and a weighted gene co-expression network analysis (WGCNA), were able to identify gene expression networks that were associated with reduced drinking (Ferguson et al. 2014). Cell-type enrichment analysis showed that both fenofibrate and tesaglitazar targeted amygdala GABAergic interneurons in a coordinated manner while the nonselective PPAR agonist bezafibrate did not. Interestingly, fenofibrate and tesaglitazar both upregulated neuropeptide and dopaminergic signaling genes in the amygdala (including *Avp* [encoding vasopressin], *Npy*, and *Pdyn* [encoding dynorphin]), suggesting that these drugs may be acting in a manner independent of their anti-inflammatory effects to regulate drinking.

The PPARγ agonist pioglitazone has also been shown to be effective at reducing alcohol relapse in rats induced by the pharmacological stressor yohimbine (Stopponi et al. 2013). Interestingly, the opioid antagonist naltrexone (which is FDA-approved for AUD) reduces cue-induced reinstatement of alcohol seeking but has no effect on stress-induced reinstatement of alcohol seeking. When naltrexone and pioglitazone are given together, however, both relapse behaviors are reduced (Stopponi et al. 2013), suggesting that these drugs may work in independent, complementary manners to reduce alcohol relapse risk. In addition to regulating drinking behaviors, pioglitazone has also been shown to be protective against alcohol neurotoxicity (Kane et al. 2011; Tajuddin et al. 2014), suggesting that PPAR agonists may be effective treatments for both AUD and fetal alcohol spectrum disorder (FASD). Indeed, pioglitazone treatment in neonatal C57BL/6J mice blocked ethanol-induced neuroinflammatory cytokine and chemokine expression and microglial activation (Drew et al. 2015). Both fenofibrate and pioglitazone are FDA-approved and are currently being used clinically to improve metabolism and decrease inflammation for a range of conditions, including insulin resistance (Shah and Mudaliar 2010) and cardiovascular disease (Rosenson et al. 2012). Patients with AUD may need to be closely monitored on these drugs, given the rare, but serious, potential side effect of liver disease.

2.3 Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors are activated by various immunological stimuli and work to amplify inflammatory responses. Since their discovery in the immune system, NF-κB factors have been found in a range of cell types (including neurons and glial cells). NF-κB consists of a family of five subunit proteins (p50, p65, p52, RelB, and c-Rel) that function as dimers. The dimers formed by these subunits are specific to cell type and developmental stage, and lend great specificity to downstream targets and function (Perkins 1997). Generally speaking, the p65/p50 heterodimer activates gene transcription (Li et al. 1994) and is the major NF-κB complex in the adult rodent brain (Yakovleva et al. 2011), while the p50 homodimer represses transcription (Guan et al. 2005). These dimers can be found in the cytoplasm under basal conditions bound to inhibitor κB (IκB) proteins. Upon immune activation, IκB is phosphorylated by the IκB kinase

(IKKβ) and targeted for degradation, allowing NF-κB to translocate to the nucleus and regulate gene transcription (Fig. 1c). Although NF-κB is classically activated by immunological stimuli, it can also be activated by glutamate (Guerrini et al. 1995). Activation of NF-κB by synaptic transmission is dependent, at least partially, on calcium/calmodulin-dependent protein kinase II (CAMKII) activation (Meffert et al. 2003). NF-κB activity can also be regulated by additional neurotransmitter systems implicated in addiction. Stimulation of dopamine D2 receptors increases, while stimulation of D1 receptors decreases, NF-κB activity (Takeuchi and Fukunaga 2003). Opioid receptors have also been shown to activate NF-κB. Acute and long-term administration of a μ-opioid receptor agonist in primary cultures of cortical neurons increased NF-κB activity, an effect that was abolished by concurrent treatment with naloxone (Hou et al. 1996).

NF-κB activation has also been associated with alcohol dependence. Polymorphisms in the p50 protein precursor gene *NFKB1* are correlated with an increased risk for developing an AUD, especially in individuals with an early onset of alcoholism (Edenberg et al. 2008). The brains of chronic alcoholics exhibit dysregulation in the NF-κB system, with reduced expression of the p50 homodimer and the p65 subunit in the dorsal prefrontal cortex (Okvist et al. 2007). Because the p50 homodimer is largely responsible for inhibiting transcription, its downregulation is associated with increased transcription of over 50 of its target genes in alcoholics (Okvist et al. 2007). The timing and dose of alcohol exposure plays a large role in determining what effect it will have on the NF-κB system. Acute alcohol exposure in C57BL/6J mice results in an upregulation of NF-κB activity, while chronic treatment downregulates its activity (Rulten et al. 2006). As an amplifier of inflammatory responses, NF-κB has been shown to regulate alcohol-induced neurotoxicity. Binge alcohol exposure activates microglia, increases NF-κB binding to DNA, and results in neurotoxicity in Sprague-Dawley rats (Crews et al. 2006). Furthermore, alcohol-induced neurotoxicity can be attributed to the activation of a number of pro-inflammatory genes by NF-κB (Zou and Crews 2010).

Anti-inflammatory compounds may be a promising strategy for manipulating the NF-κB system. Resveratrol, a natural polyphenol, prevents the acute ethanol-induced upregulation of NF-κB, decreases ethanol-induced pro-inflammatory gene transcription, and increases cognitive performance in rodents (Tiwari and Chopra 2013b). Similar therapeutic effects were found upon treatment with curcumin (Tiwari and Chopra 2013a), a biomolecule found in turmeric with well-defined anti-inflammatory pathways associated with inhibition of NF-κB (Shakibaei et al. 2007; Singh and Aggarwal 1995). Administration of the antioxidant butylated hydroxytoluene (BHT) can prevent NF-κB activation, neural damage, and pro-inflammatory gene induction that occur with ethanol exposure (Zou and Crews 2010). Specifically targeting the NF-κB pathway, the IKKβ inhibitors TPCA-1 and sulfasalazine (which prevent NF-κB from translocating to the nucleus) were able to reduce ethanol drinking in mice (Truitt et al. 2016) (Table 2). Sulfasalazine is an FDA-approved anti-inflammatory agent that is commonly used for the management of rheumatoid arthritis (Meier et al. 2013). Delayed liver toxicity may

be a serious, albeit reversible, side effect of sulfasalazine in patients with AUD (Masood et al. 2016). The occurrence of such liver toxicity is rare (<1%) in the general population and is associated with a slowed acetylation of sulfasalazine metabolites (Tanigawara et al. 2002). Currently, it is not known whether patients with AUD would be at an increased risk for sulfasalazine-induced liver toxicity.

2.4 Glucocorticoid Receptors

The connection between stress and alcohol use has long been recognized. Stress has been shown to escalate drinking in nondependent and dependent populations (Nash and Maickel 1985; Russell et al. 2017; Spanagel et al. 2014), individuals with a family history of alcohol dependence exhibit increased stress responsivity (Uhart et al. 2006), and vulnerability to stress is a reliable indicator of relapse in alcohol-dependent individuals (Brown et al. 1995; Witkiewitz 2011). There are many targets in the physiological stress pathway that may contribute to stress-induced drinking (including corticotropin releasing factor), yet the importance of glucocorticoids has increasingly been recognized (Nash and Maickel 1988; Fahlke et al. 2000).

The hypothalamic–pituitary–adrenal (HPA) axis is the body's primary stress response pathway and is responsible for the release of cortisol (in humans) or corticosterone (in rodents), herein referred to as CORT, from the adrenal glands. CORT binds to two receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). CORT has a tenfold higher affinity for MRs than for GRs, which allows MR occupancy to occur under basal conditions, and therefore restrict CORT levels at baseline via a negative feedback loop (Rupprecht et al. 1993). In contrast, GR occupancy occurs under conditions of high CORT release and is therefore largely responsible for facilitating recovery after a stressor, via negative feedback in the hypothalamus. Additionally, GRs help to promote memories of stressful events by increasing AMPA receptor expression, and thereby strengthening glutamatergic signaling, in the hippocampus and prefrontal cortex (Joels et al. 2012). The ratio of MR: GR functionality may confer resilience (when high) or vulnerability (when low) to a host of psychiatric conditions (ter Heegde et al. 2015). Under basal conditions, GRs are bound to chaperone proteins in the cytoplasm. Upon ligand binding, GR translocates to the nucleus and binds to glucocorticoid response elements (GREs) on the DNA that are often distal to the promoter region of target genes (Reddy et al. 2009) (Fig. 1d). GRs can also bind to noncanonical binding sites on DNA, interact indirectly with DNA via a tethered mechanism with other transcription factors, and interact synergistically with neighboring transcriptional regulatory proteins at combinatorial binding sites on the DNA (Ratman et al. 2013). These varied mechanisms allow for incredible complexity in the downstream transcriptional effects of GRs.

Importantly, alterations in GR expression and activity have been linked to alcohol abuse risk. Genetic polymorphisms in the GR gene (*NR3C1*) are associated with age of onset of alcohol use and abuse, a phenotype strongly correlated with risk of developing an AUD (Desrivieres et al. 2011). Individuals with alcohol dependence also exhibit a delayed and/or blunted hormonal response to a pharmacological

stressor, suggesting dysregulation of the HPA axis with heavy alcohol use (Wand and Dobs 1991). Alcohol can alter CORT and GR expression in such a way as to promote intake. Chronic intermittent ethanol exposure in rats increased peak CORT levels, transiently decreased GR signaling in the medial prefrontal cortex (mPFC) during early withdrawal, and then increased GR signaling during protracted abstinence, an effect accompanied by reinstatement of ethanol seeking (Somkuwar et al. 2017). Similarly, acute alcohol withdrawal in rats produced decreases in GR expression in other regions of the brain critical to stress/alcohol pathways, including the nucleus accumbens and bed nucleus of the stria terminalis, whereas protracted abstinence led to increased GR expression in these brain areas and escalated compulsive alcohol intake (Vendruscolo et al. 2012). CORT can also contribute to alcohol-induced neurodegeneration. Chronic alcohol exposure in adrenalectomized rats given high levels of CORT showed exacerbation of neurodegeneration, while low-dose CORT (commensurate with basal CORT levels) did not exacerbate alcohol neurotoxicity (Cippitelli et al. 2014). CORT acutely suppresses the immune system, but repeated exposures to CORT have been shown to activate microglia in mice, a response driven by GR activation (Nair and Bonneau 2006). In this way, alcohol may increase pro-inflammatory responses in the brain via direct mechanisms involving NF-κB signaling (as mentioned earlier in this chapter) as well as via GR activation.

Inhibition of GRs can reverse many of these alcohol phenotypes. Chronic treatment with mifepristone, a nonselective GR antagonist, prevented dependence-induced escalations in drinking and compulsive responding for alcohol exhibited during protracted abstinence in rats (Vendruscolo et al. 2012) (Table 2). Interestingly, mifepristone reduced alcohol intake in dependent, but not nondependent rats, suggesting that the GR dysregulation that occurs with chronic alcohol exposure is a unique risk factor for escalated use (Vendruscolo et al. 2015). Mifepristone may also prove to aid in the treatment of symptoms associated with ethanol withdrawal. Rats treated with mifepristone showed a dose-dependent reduction in several withdrawal-related behaviors, including tremor and tail rigidity (Sharrett-Field et al. 2013), and a single treatment of mifepristone in mice reduced the cognitive deficits observed during withdrawal (Jacquot et al. 2008). Daily doses of mifepristone have also been shown to attenuate alcohol-induced hippocampal neurodegeneration in rats in a dose-dependent manner (Cippitelli et al. 2014). Although most work with mifepristone has been conducted in animals, preliminary research in humans has shown promising results. Just 1 week of mifepristone treatment in alcohol dependent human subjects reduced both alcohol craving and consumption (Vendruscolo et al. 2015).

Mifepristone shows great promise as a treatment for AUD, but there are limitations to its use. In addition to blocking GRs, mifepristone is also a potent antagonist of the progesterone receptor and is most commonly used clinically to terminate pregnancies. As such, female patients receiving mifepristone can experience vaginal bleeding due to endometrial thickening. Other side effect profiles are low, even with chronic treatment. Long-term, low-dose mifepristone used to treat uterine fibroids resulted in no significant side effects (Kapur et al. 2016). At much higher doses, mifepristone has been used to treat Cushing's syndrome, which is characterized by chronic, excessive exposure to glucocorticoids. At these high doses, mifepristone can cause more serious side effects, including hypertension, hypokalemia, and edema (Cuevas-Ramos et al. 2016).

Taken together, these results suggest that GR activation is critical for both the development and maintenance of AUD, and disrupting GR signaling using mifepristone may be promising for preventing relapse and treating withdrawal symptoms in alcohol-dependent individuals.

3 Epigenetic Modifiers

3.1 DNA Methyltransferases

In mammals, DNA methylation is catalyzed by three DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B (Day et al. 2015). These enzymes add a methyl group to the fifth carbon position of the cytosine (5mC) found adjacent to guanine (as cytosine-phosphate-guanine dinucleotides, or CpGs) (Zovkic et al. 2013), using the methyl donor S-adenosyl methionine (SAM). The genome contains regions that are rich in CpGs, known as CpG islands, which are often found in gene regulatory or promoter regions. The methyl-binding domain proteins (e.g., MeCP2) directly interact with 5mC, that then recruit chromatin-modifying proteins and transcriptional repressor complexes to the DNA (Zovkic et al. 2013). Thus, DNAm is normally associated with repression of gene expression (Fig. 2).

DNAm in the brain plays an important role in learning and memory (Zovkic et al. 2013), and evidence is accumulating that DNAm is also important in AUD (Tulisiak et al. 2017). Increased expression of DNMT1 protein and *DNMT3a* and *3b* genes in rodent brains, and decreased expression of *DNMT3A* and *3B* genes in human blood samples, have been observed after chronic ethanol exposure (Barbier et al. 2015; Bonsch et al. 2006; Warnault et al. 2013; Qiao et al. 2017). The difference in *DNMT3A* and *3B* expression between brain and blood samples may be due to species differences (rat vs. human), different tissue-specific responses to ethanol, or duration or timing of alcohol exposure. Nonetheless, changes in DNMTs after chronic ethanol exposure indicate that DNAm might be altered by alcohol.

Additional circumstantial evidence that DNAm is altered by alcohol exposure has come from an analysis of postmortem cerebellum from human subjects with AUD compared with that of control subjects. In this study, the authors measured increased transcript levels of enzymes involved in the one-carbon metabolism pathway, which generates the methyl donor SAM (Gatta et al. 2017). Correlated with this was an increase in the ratio of SAM to s-adenosylhomocysteine (SAH) in the cerebellum, which would increase the activity of DNMTs (Auta et al. 2017; Gatta et al. 2017). Similar changes in the SAM/SAH ratio occurred in rat cerebellum after chronic ethanol drinking, indicating that the changes are induced by alcohol (Auta et al. 2017). Finally, mRNA levels of an enzyme (tet methylcytosine dioxygenase 1, *TET1*) that removes methyl groups from cytosines was decreased in the cerebellum of subjects with AUD (Gatta et al. 2017). Together, these results indicate that changes in enzymes that regulate DNAm are associated with chronic alcohol exposure and suggest that there might be increased total DNAm in the brain. However, more detailed studies described below, examining differentially methylated regions after chronic alcohol use, suggest

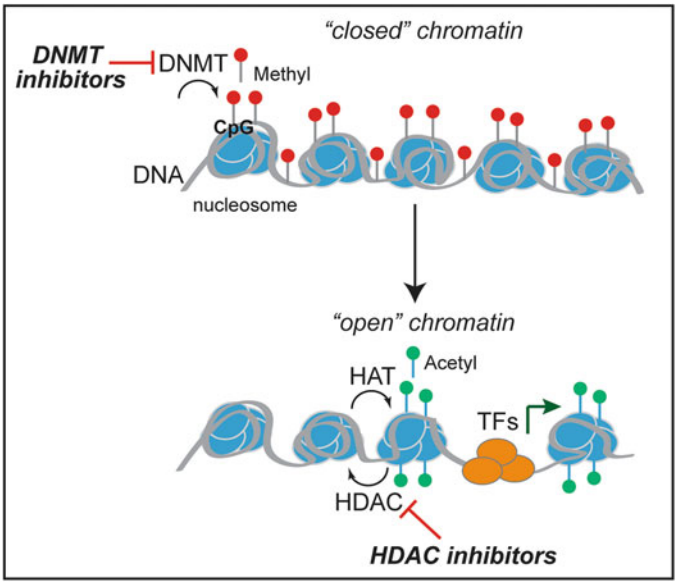

Fig. 2 Simplified diagram of epigenetic modifications and epigenetic enzyme targets for intervention for AUD. DNA (gray line) is wrapped around histone octamers to form the nucleosome (shown in blue), the basic unit of chromatin. Top panel: DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and is associated with condensed chromatin and repression of gene expression. DNMT inhibitors reduce alcohol consumption in animal models of AUD. Bottom panel: histone acetylation is catalyzed by the histone acetyltransferases (HATs) and is generally associated with open chromatin, increased transcription factor availability, and activation of gene expression. Removal of acetyl groups from histones is achieved by the histone deacetylases (HDACs). HDAC inhibitors reduce alcohol consumption in animal models of AUD

that specific genomic locations associated with excessive drinking can be either hypo- or hypermethylated.

More explicit evidence that changes in DNAm are associated with alcohol use was first provided by analysis of genomic DNA from blood cells of alcoholic patients, with increased total DNAm associated with alcoholism (Bonsch et al. 2004). Another study found decreased DNAm at repetitive DNA elements (Alu) associated with alcohol use in lymphocytes from healthy individuals (Zhu et al. 2012). Candidate gene approaches in blood cells have demonstrated associations between increased DNAm and AUD at the promoters for alpha synuclein (*SNCA*), homocysteine-induced endoplasmic reticulum protein (*HERPUD1*), serotonin transporter (*SLC6A4*), monoamine oxidase A (*MAOA*), prodynorphin (*PDYN*), and aldehyde dehydrogenase 2 (*ALDH2*) (Bleich et al. 2006; Bonsch et al. 2005; Philibert et al. 2008a, b; D'Addario et al. 2017; Pathak et al. 2017). More recently, genome-wide profiling of methylated regions in whole blood cells or human lymphoblast cell lines has demonstrated several significant differentially methylated regions or differentially methylated cytosines associated with high levels of alcohol drinking or dependence (Philibert et al. 2012; Clark et al. 2015; Liu et al. 2016; Zhang et al. 2013; Zhao et al. 2013). In another study

by Philibert et al. (2014), differentially methylated cytosines were identified in peripheral mononuclear cell DNA from subjects with heavy alcohol use compared with controls. Genome-wide DNAm was measured in the AUD subjects as they entered a treatment facility and ~25 days later, during abstinence. Many of the differentially methylated cytosines identified between controls and alcohol-dependent subjects were reversed during abstinence to levels similar to controls. Similarly, Bruckmann et al. (2017) found that some of the differentially methylated cytosines identified in CD3+ T cells between healthy controls and alcohol-dependent patients reverted back to control levels after abstinence, demonstrating a potential causal role for alcohol in changing DNAm in blood cells.

Studies in blood, lymphoblast cell lines, or other peripheral tissues may not represent the DNAm patterns in the brain associated with alcohol use. A few studies have found similar changes occurring in postmortem brain and peripheral tissues. Notably, DNAm in the promoter for the gene encoding the delta subunit of the GABA-A receptor (*GABRD*) was increased in the cerebellum of human subjects with AUD compared with controls, similar to what has been observed in lymphocytes (Gatta et al. 2017; Liu et al. 2016). The increase in DNAm in the promoter of *GABRD* in the cerebellum was associated with decreased *GABRD* expression (Gatta et al. 2017). In the precuneus brain region from AUD subjects, 244 hypomethylated and 188 hypermethylated regions were associated with alcohol dependence (Hagerty et al. 2016). These differentially methylated cytosines overlapped with those found in buccal (cheek) cells collected from the same subjects (Hagerty et al. 2016). Together, these studies indicate that alterations in DNAm in the brain occur with chronic alcohol use and that some of these changes are similar to those occurring in peripheral tissues, suggesting that DNAm changes at particular genetic loci could be used as a diagnostic measure for AUD and possibly treatment response.

Additional genome-wide analysis of DNAm in different brain regions in humans, and monkeys has been performed in order to identify both region-specific changes in DNAm and potential new candidate genes for AUD. Analysis of prefrontal cortex tissue from AUD and control subjects found 1,812 differentially methylated cytosines mapping to 1,099 genes that were significantly associated with AUD (Wang et al. 2016). In rhesus macaques, differentially methylated regions in the nucleus accumbens discriminated abstinent monkeys from low/binge drinkers and heavy/very-heavy drinkers and were located in genes encoding synaptic, cell signaling, and receptor trafficking mediators (Cervera-Juanes et al. 2017a, b) that could, in theory, be targets for pharmacological intervention.

Three pharmacological agents have been used to inhibit DNMTs and determine the effect on behaviors related to AUD in animal models: the nucleoside analogs 5-azacytidine (azacytidine), 5-aza-2′deoxycytidine (decitabine), and RG108, a non-nucleoside DNMT inhibitor (Table 3). Mice treated with azacytidine or decitabine reduced their ethanol intake in intermittent access procedures that model binge-like drinking (Warnault et al. 2013; Ponomarev et al. 2017). In alcohol-dependent rats, infusion of RG108 into the cerebral ventricles resulted in decreased alcohol intake after a three-week period of forced abstinence when compared with vehicle-treated rats (Barbier et al. 2015). Similarly, infusion of decitabine into the mPFC of

Table 3 Compounds that act on epigenetic enzymes and reduce ethanol consumption

Compound	Target	Species tested	Approved for clinical use	References
Azacytidine	DNMT1/3a/3b	Mice	Yes[a]	Warnault et al. (2013)
Decitabine	DNMT1/3a/3b	Mice, rats	Yes[a]	Ponomarev et al. (2017) and Qiao et al. (2017)
RG108	DNMT1/3a/3b	Rats	No	Barbier et al. (2015)
Trichostatin A (TSA)	Class I, II, IV HDACs	Rats, mice	No	Warnault et al. (2013), Pandey et al. (2015), and Sakharkar et al. (2014)
Suberanilohydroxamic acid (SAHA, Vorinostat)	Class I, II, IV HDACs	Mice, rats	Yes[b]	Warnault et al. (2013)
Valproic acid (VPA)	HDAC1/2	Rats	Yes[c]	Al Ameri et al. (2014)
Sodium butyrate (NaB)	Class I, IIa HDACs	Rats	No	Simon-O'Brien et al. (2015)
MS-275 (Entinostat)	HDAC1/3	Rats, mice	No	Warnault et al. (2013), Simon-O'Brien et al. (2015), and Jeanblanc et al. (2015)

[a]Approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia
[b]Approved for the treatment of cutaneous T-cell lymphoma
[c]Approved for the treatment of seizures, mania, bipolar disorder, and to prevent migraines

chronically drinking rats decreased ethanol intake but increased anxiety-like behavior (Qiao et al. 2017). The timing of administration appears to be important for the ability of DNMT inhibitors to reduce ethanol drinking. When ethanol-dependent mice were given intracerebroventricular azacytidine during the induction of dependence (immediately before ethanol vapor exposure), they subsequently consumed more ethanol in a 2BC test (Qiang et al. 2014). However, generally the evidence indicates that DNMT inhibitors reduce ethanol intake in rodent models of binge and dependence-induced drinking.

Azacytidine and decitabine are FDA-approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia. Both of these drugs have high toxicity (Gnyszka et al. 2013) and serious side effects of these drugs include increased bruising, bleeding, and infection. Azacytidine is contraindicated for individuals with liver tumors, and those with liver and kidney disease should be monitored carefully. As a result, these drugs should not be used for those individuals with alcohol-associated liver disease. A newer nucleoside analog, zebularine, has less toxicity (Gnyszka et al. 2013) but has not yet been tested in animal models of AUD. In summary, DNMT inhibitors represent a promising pharmacotherapeutic approach to treat AUD, but newer generation compounds, such as zebularine and RG-108, which are not yet FDA-approved, require further investigation.

3.2 Histone Deacetylases and Histone Acetyltransferases

DNA wrapped around a histone octamer forms the nucleosome, an integral building block of chromatin structure. Changes in the acetylation state of histone tails are intimately involved in chromatin remodeling and transcriptional alterations. HATs are enzymes that add acetyl groups to lysine residues on histone proteins, while histone deacetylases (HDACs) remove these acetyl groups (Elvir et al. 2017; Haberland et al. 2009) (Fig. 2). Generally, HATs activate transcription while HDACs repress it, although there are exceptions (Haberland et al. 2009; Sacconnay et al. 2016). The mammalian genome encodes 11 "classical," zinc-dependent HDACs that are categorized into four families (class I: HDAC1, 2, 3, and 8; class IIa: HDAC4, 5, 7, and 9; class IIb: HDAC6 and 10; and class IV: HDAC11). A separate family of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases, called sirtuins (or class III HDACs), comprises 7 members (SIRT1-7) (Sacconnay et al. 2016). Recent evidence shows alterations in histone acetylation/deacetylation and chromatin structure in several psychiatric disorders, suggesting that these processes may underlie motivated behaviors, including drug addiction (Elvir et al. 2017; Pena et al. 2014).

Ethanol changes the acetylation state of histones after acute and chronic exposure and during withdrawal. Acute ethanol exposure led to changes in histone H3 and H4 acetylation in the amygdala, hippocampus, and cortex (D'Addario et al. 2013; Finegersh and Homanics 2014; Pandey et al. 2008; Sakharkar et al. 2012). Chronic ethanol exposure and/or withdrawal altered acetylation of histones H3 and H4 in the amygdala, ventral tegmental area, cortex, nucleus accumbens, dorsal striatum, and hippocampus (Arora et al. 2013; Bohnsack et al. 2017; Botia et al. 2012; D'Addario et al. 2013; Dominguez et al. 2016; Finegersh et al. 2015; Qiang et al. 2011; Shibasaki et al. 2011; Simon-O'Brien et al. 2015; You et al. 2014; Pandey et al. 2008). In general, the changes in total histone H3 and H4 acetylation induced by acute alcohol exposure appear to be the opposite to those of withdrawal from chronic alcohol exposure. However, this depends on the brain region. Alcohol-induced changes in acetylation in the promoter regions of specific genes such as *GABRA1* (Arora et al. 2013; Bohnsack et al. 2017), *PDYN* (D'Addario et al. 2013), *PNOC* (D'Addario et al. 2013), *BDNF* (You et al. 2014), *ARC* (You et al. 2014), *NPY* (Pandey et al. 2008; Sakharkar et al. 2012), and *GRIN2B* (NR2B) (Qiang et al. 2011, 2014) were associated with changes in gene expression, which has important consequences for behaviors such as anxiety during withdrawal (Pandey et al. 2008) and ethanol consumption (Qiang et al. 2014).

Treatment with HDAC inhibitors is effective in reducing ethanol intake in multiple models of AUD (Table 3). Mice treated with Trichostatin A (TSA) or Vorinostat (SAHA) (inhibitors of class I, II, and IV HDACs) consumed less ethanol in a limited-access binge-drinking test, and rats treated with SAHA also self-administered less ethanol in an operant task and exhibited reduced alcohol-seeking behavior (Warnault et al. 2013). Treatment with the HDAC1/2 inhibitor valproic acid (VPA) decreased 2BC ethanol consumption and preference by rats and also blocked ethanol reward, as measured in the conditioned place preference test (Al Ameri et al. 2014). In the Warnault and Al Ameri studies, the effect of the HDAC inhibitors on histone acetylation in the

brain was not measured, but Warnault et al. did observe a decrease in total histone H4 acetylation in the nucleus accumbens after binge drinking by mice and ethanol self-administration by rats (Warnault et al. 2013). In summary, these studies indicate that TSA, SAHA, and VPA decrease binge-like drinking or alcohol reward/reinforcement-related behavioral measures.

Heavy-drinking rats treated with the HDAC1/3 inhibitor Entinostat (MS-275) also self-administered less ethanol and exhibited reduced relapse (reinstatement) to ethanol seeking after a period of abstinence, an effect that was associated with increased histone H4 acetylation in the nucleus accumbens and dorsolateral striatum (Jeanblanc et al. 2015). Treatment with the nonselective HDAC class I and IIa inhibitor sodium butyrate (NaB), or MS-275, reduced operant ethanol self-administration by ethanol-dependent rats, but these compounds did not affect responding for ethanol in nondependent animals (Simon-O'Brien et al. 2015). In addition, NaB treatment reduced ethanol drinking by rats in an intermittent access 2BC drinking experiment and prevented the escalation of ethanol intake that occurs after alcohol deprivation (Simon-O'Brien et al. 2015). In this study, histone H3 lysine 9 acetylation varied between ethanol-dependent and nondependent rats depending on the brain region, and NaB treatment did not uniformly increase histone acetylation in all brain regions as might be predicted of an HDAC inhibitor. For instance, in the prefrontal cortex of ethanol-dependent rats, NaB decreased histone H3 acetylation (Simon-O'Brien et al. 2015). This demonstrates that there are complicated region-specific alterations in total histone acetylation after alcohol exposure. In the studies described above in which HDAC inhibitors were tested for their role in ethanol consumption, histone acetylation at specific gene promoters was not examined. Identifying these genes and the brain regions in which they act to regulate alcohol drinking is a clear area for future research. Nonetheless, these studies demonstrate that NaB and MS-275 treatment can prevent relapse to alcohol drinking in animals that are alcohol-dependent.

Ethanol withdrawal causes anxiety, promotes relapse to drinking, and is associated with several changes in the amygdala: increased nuclear HDAC activity, decreased acetylated histones, decreased expression of the HAT CBP (CREB binding protein), decreased expression of *Npy*, *Bdnf*, and *Arc*, and decreased dendritic spine density (Pandey et al. 2008; You et al. 2014). Treatment of rats with TSA reversed ethanol withdrawal-induced anxiety and the epigenetic, gene expression, and structural changes observed in the amygdala (Pandey et al. 2008; You et al. 2014). HDAC inhibitors are also effective in a genetic model of AUD, the alcohol-preferring P rat. P rats have higher anxiety and ethanol intake compared with alcohol non-preferring NP rats, higher levels of nuclear HDAC activity, more HDAC2 protein, decreased acetylated histones, and decreased NPY protein in the amygdala (Moonat et al. 2013; Sakharkar et al. 2014). Treatment of P rats with TSA, or with HDAC2 siRNA in the amygdala, reduced anxiety and ethanol intake and normalized the associated epigenetic alterations and NPY levels (Moonat et al. 2013; Sakharkar et al. 2014). Since anxiety is associated with an increased risk of relapse in alcohol-dependent individuals (Schellekens et al. 2015), preventing the development of withdrawal-induced anxiety through the use of HDAC inhibitors may be a promising method for encouraging abstinence in recovering alcoholics.

Changes in histone acetylation can persist long after the initial exposure to alcohol. Adolescence is a period of brain development in which synaptic structural modifications and changes in neural plasticity are occurring. Exposure of animals to alcohol during adolescence leads to long-lasting alterations in histone acetylation that persist into adulthood and these changes are associated with increased anxiety-like behavior and high levels of ethanol consumption (Kokare et al. 2017; Pandey et al. 2015; Pascual et al. 2009, 2012; Sakharkar et al. 2016). Treatment of adult rats with TSA after they had been exposed to ethanol during adolescence normalized the high levels of anxiety, ethanol intake, and alcohol-induced histone acetylation and gene expression changes (Pandey et al. 2015; Sakharkar et al. 2016). These studies suggest that the persistent histone acetylation changes associated with alcohol exposure during adolescence can be reversed by treatment with HDAC inhibitors in adulthood and attenuate pathological anxiety and excessive drinking.

Taken together, HDAC inhibitors have been found generally to decrease excessive ethanol-drinking and ethanol-seeking behavior in rodents. However, there are a few exceptions. Wolstenholme et al. found that treatment of mice with TSA increased voluntary 2BC ethanol intake, and Qiang et al. demonstrated that treatment of mice with TSA during exposure to ethanol vapor subsequently led to increased ethanol drinking (Qiang et al. 2014; Wolstenholme et al. 2011). In addition, Ponomarev et al. found that SAHA had no effect on either a binge-drinking test or in a chronic intermittent drinking protocol (Ponomarev et al. 2017). These results indicate that the timing of administration of HDAC inhibitors may be important when considering them for AUD treatment.

SAHA is FDA-approved for the treatment of cutaneous T-cell lymphoma. Serious side effects include increased risk of developing blood clots, increased bruising, bleeding, or susceptibility to infection, and increased blood sugar levels. There are no known interactions with light alcohol drinking. SAHA might be a viable option to move forward in clinical studies. VPA has long been prescribed for the treatment of seizures and bipolar disorder. Serious side effects of VPA include blistering, peeling, or red skin rash, confusion, memory problems, suicidal thoughts, and depression. VPA can also cause liver problems, pancreatitis, and thrombocytopenia. As also noted for the DNMT inhibitors (azacytidine and decitabine), liver disease may preclude the use of VPA in alcohol-dependent patients. Finally, it should be mentioned that systemic administration of DNMT and HDAC inhibitors could have potentially deleterious effects on gene expression in several tissues because they block the activity of enzymes expressed throughout the body.

In terms of treating AUD patients with compounds targeting epigenetic modifiers, the aforementioned animal studies may provide insight into compounds that may work best for different subtypes of alcoholism or at different phases of the addiction cycle. For instance, DNMT inhibitors might be useful in decreasing binge-like drinking, and a non-nucleoside DNMT inhibitor such as RG-108 could also be effective in treating alcohol-dependent individuals during abstinence to prevent relapse. SAHA may be effective in reducing binge-like drinking and also anxiety during alcohol withdrawal. Finally, the HDAC1/3 inhibitor MS-275 appears to have limited toxicity (Subramanian et al. 2010), is currently in clinical trials for cancer treatment, and may

be another option for treating alcohol-dependent patients during abstinence to prevent relapse.

4 Conclusions and Future Directions

Several small molecule compounds have been developed that target transcriptional regulators and epigenetic enzymes that have shown effectiveness in reducing alcohol drinking in several rodent models of AUD. One of these compounds, mifepristone, has shown promising results in a human laboratory study and is already FDA-approved for other conditions. Future studies should focus on translating the findings of other compounds to clinical studies to determine if they can reduce excessive alcohol drinking in human subjects with AUD. Several new promising candidates exist, including PDE4 inhibitors, PPARα/γ agonists, HDAC inhibitors, and DNMT inhibitors. A focus on repurposing those compounds that are already FDA-approved for other conditions may be an efficient mechanism to getting these into clinical use for those suffering from AUD.

Acknowledgements A.W.L. is supported by the National Institute on Alcohol Abuse and Alcoholism (INIA U01 AA020912 and the Center for Alcohol Research in Epigenetics P50 AA022538).

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Part V

Pharmacotherapy

Advancing Pharmacotherapy Development from Preclinical Animal Studies

Mark Egli

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Abstract

Animal models provide rapid, inexpensive assessments of an investigational drug's therapeutic potential. Ideally, they support the plausibility of therapeutic efficacy and provide a rationale for further investigation. Here, I discuss how the absence of clear effective-ineffective categories for alcohol use disorder (AUD) medications and biases in the clinical and preclinical literature affect the development of predictive preclinical alcohol dependence (AD) models. Invoking the analogical argument concept from the philosophy of science field, I discuss how models of

The original version of this chapter was revised. A correction to this chapter is available at https://doi.org/10.1007/164_2018_191.

M. Egli (✉)
Division of Neuroscience and Behavior, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD, USA
e-mail: mark.egli@nih.gov

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_85

excessive alcohol drinking support the plausibility of clinical pharmacotherapy effects. Even though these models are not likely be completely discriminative, they are sensitive to clinically effective medications and have revealed dozens of novel medication targets. In that context, I discuss recent preclinical work on GLP-1 receptor agonists, phosphodiesterase inhibitors, glucocorticoid receptor antagonists, nocioceptin agonists and antagonists, and CRF1 antagonists. Clinically approved medications are available for each of these drug classes. I conclude by advocating a translational approach in which drugs are evaluated highly congruent preclinical models and human laboratory studies. Once translation is established, I suggest the burden is to develop hypothesis-based therapeutic interventions maximizing the impact of the confirmed pharmacotherapeutic effects in the context of additional variables falling outside the model.

Keywords
Alcohol use disorder · Pharmacotherapy · Preclinical models · Translational research

Abbreviations

AD	Alcohol dependence
ADE	Alcohol deprivation effect
AUD	Alcohol use disorder
BAC	Blood alcohol concentration
BNST	Bed nucleus of the stria terminalis
cAMP	Cyclic adenosine monophosphate
CeA	Central nucleus of the amygdala
cGMP	Cyclic guanosine monophosphate
CIE	Chronic intermittent ethanol
CPP	Conditioned place preference
GR	Glucocorticoid receptor
HDID	High drinking in the dark
HPA	Hypothalamus-pituitary-adrenal
LC	Locus coeruleus
N/OFQ	Nociceptin/orphanin-FQ
NAc	Nucleus accumbens
PFC	Prefrontal cortex
PVN	Paraventricular nucleus
RCT	Randomized controlled trials
SA	Self-administration
VTA	Ventral tegmental area

1 Orientation

Investigational drug HBJ714 is effective in preclinical alcohol dependence (AD) models; can we predict that HBJ714 will be an effective alcohol use disorder (AUD) pharmacotherapy in humans? To answer, we must consider: What is a

clinically effective AUD medication? Do clinically effective medications have similar effects and do clinically ineffective drugs have distinct effects when tested in the preclinical AD models?

1.1 What Is Clinically Effective?

Can we distinguish clinically effective from clinically ineffective AUD medications? Table 1 shows medications that have been tested in double-blind randomized, placebo-controlled studies ($N \geq 10$ in each treatment arm; treatment $\geq$2 months). The table is illustrative, not exhaustive, and does not attempt definitive conclusions regarding efficacy (see Litten et al. 2016; Litten et al. 2018, this volume for recent reviews). Despite negative clinical trial outcomes or controversies, I accept naltrexone, nalmefene and acamprosate as effective medications because they have met universal efficacy and safety standards via FDA or comparable regulatory agency approval. Disulfiram is not considered due to its distinct mechanism and its failure to show efficacy in blinded RCTs compared to controls (Skinner et al. 2014; Yoshimura et al. 2014).

Impressions about clinical efficacy from the literature must be taken cautiously as they are shaped by the order of negative and positive outcomes. Early negative outcomes diminish interest in further testing. Had negative naltrexone (Krystal et al. 2001) or acamprosate (e.g., Anton et al. 2006) RCTs been the first reported, fewer additional trials – including those which proved to be positive – would likely have been performed. Negative outcomes carry less weight if they follow positive findings (e.g., the continued interest in baclofen after early positive findings despite many subsequent negative RCTs; see Litten et al. 2016).

Table 1 shows that medications do not fall clearly into dichotomous effective-ineffective categories. Some, like naltrexone, ondansetron, or gabapentin, demonstrate efficacy in multiple independent RCTs. Others, like tiapride, flupenthixol, and levetiracetam, may even worsen clinical indications. Many more show mixed outcomes. Mapping preclinical measures to these clinical studies seems daunting.

1.2 How Do Clinically Evaluated AUD Medications Perform in Preclinical Tests?

As shown in Table 1, preclinical testing of clinically evaluated AUD drugs is uneven, unsystematic, and heavily focused on drinking or operant self-administration (SA) in nondependent rodents. Such models are sensitive to clinically effective medications, but they are also sensitive to clinically ineffective medications.

Impressions of published preclinical studies may also be biased. Heavy resource investment justifies publishing negative clinical trials; there is less incentive to publish negative preclinical studies. Published preclinical studies appear optimistic as a result (see Yardley and Ray 2017; Fig. 1). Negative preclinical tests often lead to abandonment especially in medication development contexts. Explanations for the

Table 1 Clinical trial and preclinical test outcomes for select AUD medications[a]

Medication	Mechanism of action	Primary outcome	Effects in preclinical AUD models (examples)
Level 1: Regulatory agency approval			
Naltrexone	Opioid receptor antagonist	US FDA approval 1994 *Negative multicenter trial in VA population*: Krystal et al. (2001)	Reduced drinking in P rats (Froehlich et al. 1990) Reduced SA in monkeys (Boyle et al. 1998) Reduced cue-induced reinstatement (Dayas et al. 2007) Blocked CPP (Kuzmin et al. 2003) Blocked ADE (Kuzmin et al. 2007)
Nalmefene	MOR, DOR antagonist; KOR partial agonist	EU EMA approval 2013 *Negative multisite trial* (Anton et al. 2004) Conflicting meta-analysis data (Palpacuer et al. 2015; Mann et al. 2016)	Reduced self-administration (SA) in nondependent and dependent rats (Walker and Koob 2007) Reduced self-administration in P rats (June et al. 2004)
Acamprosate	Glutamate modulator	France, approval 1989 US FDA approval 2004	Reduced drinking in HDID mice (Crabbe et al. 2017) Selectively reduced drinking in dependent rats (Le Magnen et al. 1987) Blocked CPP (McGeehan and Olive 2003) Blocked cue-induced reinstatement (Bachteler et al. 2005) Blocked ADE (Heyser et al. 2003)
Level 2: Two or more independent definitive RCT			
Ondansetron	5-HT3 antagonist	Decreased drinks/day (Sellers et al. 1994) Decreased drinks/day (Johnson et al. 2000)	Reduced SA in rats (Tomkins et al. 1995) Reduced SA in heavy drinking P more than light drinking P and Wistar rats (Lynch et al. 2011) No effect on SA in rats (Beardsley et al. 1994) Attenuated footshock reinstatement (Lê et al. 2006)
Gabapentin	GABA modulator,	Decreased drinks/day and heavy drinking days; increased % days abstinent	Reduced SA in dependent more than nondependent rats (Roberto et al. 2008)

(continued)

Table 1 (continued)

Medication	Mechanism of action	Primary outcome	Effects in preclinical AUD models (examples)
	voltage-gated Ca^{2+} channel	(Furieri and Nakamura-Palacios 2007) Increased complete abstinence; decreased heavy drinking (Mason et al. 2014)	Increased SA nondependent rats (Besheer et al. 2016)
Topiramate	Glutamate and GABAA receptor modulator	Decreased drinks/day, drinks per drinking day, % HDD; increased % days abstinent (Johnson et al. 2003) *Decreased % HDD* (Johnson et al. 2007) Increased time to first relapse, cumulative abstinence duration; decreased weeks of heavy drinking (Baltieri et al. 2008) No effect (Likhitsathian et al. 2013) Thailand *No effect on relapse to drinking* (Anthenelli et al. 2017)	Reduced stress-induced drinking in mice (Farook et al. 2009) Reduced drinking in P, but not Wistar rats (Breslin et al. 2010) Drinking in dependent mice reduced more than nondependent (Becker and Lopez unpublished)
Varenicline	α7 NAC antagonist; α4β2, α3β4, and α6β partial agonist	Decreased drinks/week (heavy smokers) (Mitchell et al. 2012b) *Decreased % HDD; drinks/day, drinks/drinking day, alcohol craving* (Litten et al. 2013b) No effect significant effects (Plebani et al. 2013) No effect %HDD; decreased craving AUDIT scores (de Bejczy et al. 2015)	No effect on SA in Wistar rats (Funk et al. 2016) Decreased SA in Wistar rats (Steensland et al. 2007) Decreased seeking, drinking in baboons (Kaminski and Weerts 2014) Reduced drinking in UChB rats (Sotomayor-Zarate et al. 2013) No effect on drinking by msP rats (Scuppa et al. 2015) Blocks ADE in P rats (Froehlich et al. 2017) No effect CPP (Gubner et al. 2014) Blocked cue reinstatement (Wouda et al. 2011)
Zonisamide	Sodium and T-type calcium channel blocker;	Decreased HDD, drinks/week (Arias et al. 2010) Decreased drinks/day, % days drinking, % days	Decreased 2-h drinking in rats and mice (Knapp et al. 2007)

(continued)

Table 1 (continued)

Medication	Mechanism of action	Primary outcome	Effects in preclinical AUD models (examples)
	GABA, Glu modulator	heavy drinking (Knapp et al. 2015)	
Level 3: Negative primary outcome, positive secondary outcomes			
ABT-436	*V1B antagonist*	*No significant effect % HDD; increased % days abstinent* (Ryan et al. 2017)	*Related compound, SSR149415, selectively reduced SA in dependent vs. nondependent rats* (Edwards et al. 2012) *SSR149415 reduced drinking in sP rats* (Zhou et al. 2011)
LY2196044	MOR, DOR, KOR antagonist/ inverse agonist	*No significant effect: % HDD, % days abstinent per month; decreased drinks/ day* (Wong et al. 2014)	Not reported
Inconclusive: mixed positive negative			
Baclofen	$GABA_B$ agonist	Increased % abstinent patients and cumulative abstinence, decreased drinks/day (Addolorato et al. 2002, 2007) No significant effect % HDD (Garbutt et al. 2010) Increased % days abstinent (Leggio et al. 2015) Increased total abstinence, cumulative abstinence (Müller et al. 2015) No significant effect % HDD; % days abstinent (Ponizovsky et al. 2015) No significant effect % abstinent patients, EtOH consumption, reduced craving (Reynaud et al. 2017)	Decreased drinking in HDID mice (Crabbe et al. 2017) Decreased SA in rats (Besheer et al. 2004) Decreased SA and drinking in baboons (Duke et al. 2014) Decreased SA in dependent rats more than nondependent rats (Walker and Koob 2007) Diminished ADE in sP rats (Colombo et al. 2003) Blocked cue reinstatement in sP rats (Maccioni et al. 2012)
Negative			
Aripiprazole	5-HT1A and D2 partial agonist	*No significant effect on % days abstinent, % subjects abstinent, drinks/drinking day* (Anton et al. 2008a)	Reduced drinking in AA rats at highest (6 mg/kg) dose (Ingman et al. 2006) Reduced drinking in rats (Nirogi et al. 2013)
Bromocriptine	D2 receptor agonist	No significant effect on drinking (Dongier et al. 1991) No significant effect on drinking, craving (Powell et al. 1995) decreased	Decreased SA in Wistar and P rats (Weiss et al. 1990) Increased drinking sweetened EtOH (Nadal et al. 1996)

(continued)

Table 1 (continued)

Medication	Mechanism of action	Primary outcome	Effects in preclinical AUD models (examples)
		craving; most with DRD2 A1 allele (Lawford et al. 1995) No significant effect on relapse (Naranjo et al. 1997)	Decreased drinking in UChB rats (Mardones and Quintanilla 1996) Decreased drinking in C57 mice (Ng and George 1994) decreased SA in rats (Cohen et al. 1998)
Flupenthixol	*D2 and 5-HT2A receptor antagonist*	*Increased relapse in males* (Wiesbeck et al. 2001)	*Not reported*
Tiapride	*D2 and D3 antagonist*	*No significant effect relapse (treatment group relapsed sooner)* (Bender et al. 2007)	*None reported*
Quetiapine	Antipsychotic	*No significant effect % HDD other drinking measures* (Litten et al. 2012) No significant effect "Type A" alcoholics, reduced DD, HDD, craving "Type B" alcoholics (Kampman et al. 2007)	No effect drinking P and HAD1 rats, dependent mice (Bell, Becker, and Lopez unpublished)
Ritanserin	5-HT2 receptor antagonist	*No significant effect on drinking or craving* (Johnson et al. 1996) No significant effect on drinking, craving or relapse (Wiesbeck et al. 1999)	No effect on drinking by cAA rats (Maurel et al. 1999)
Dexfenfluramine	SSRI	No significant effect on drinking measures (Romach et al. 2000)	Attenuated footshock reinstatement in rats (Lê et al. 2006)
Fluoxetine	SSRI	No significant effect on drinking measures (Naranjo et al. 1990) No significant effect on drinking days, drinks/day, drinks/drinking day (Kranzler et al. 1995)	Reduced SA in P rats (Murphy et al. 1988) Blocked withdrawal-induced SA in rats (Simon O'Brien et al. 2011) Increased ADE (Alén et al. 2013) Blocked footshock reinstatement (Lê et al. 1999)
Fluvoxamine	*SSRI*	*Tendency for increased relapse in treatment group* (Chick et al. 2004)	*Decreased SA in rats* (Lamb and Järbe 2001)

(continued)

Table 1 (continued)

Medication	Mechanism of action	Primary outcome	Effects in preclinical AUD models (examples)
Buspirone	5-HT1A agonist	No significant effects relapse, drinks/day (Malcolm et al. 1992) No significant effect on drinking (Kranzler et al. 1994) No significant effect on drinking (Malec et al. 1996) No significant effect on time to relapse (George et al. 1999) No significant effect on drinking (Fawcett et al. 2000)	No effect on drinking in rats (short alcohol history). Reduced drinking (longer history) (Hedlund and Wahlström 1996) Decreased drinking in rats (Hedlund and Wahlström 1999)
Memantine	NMDA receptor antagonist	No significant effects on drinking or abstinence (Evans et al. 2007)	Decreased drinking in dependent and nondependent rats (Alaux-Cantin et al. 2015) Decreased drinking in mHEP rats (Malpass et al. 2010) Decreased drinking in sP rats (Sabino et al. 2013) Decreased drinking in HAP mice (Oberlin et al. 2010) Decreased cue reinstatement in rats (Vengeliene et al. 2015) Blocked ADE (Hölter et al. 1996)
Levetiracetam	Anticonvulsant	*No significant effect % HDD* (Fertig et al. 2012) No significant effect time to relapse (Richter et al. 2012) No significant effect, increased drinking in lower drinkers (Mitchell et al. 2012a) No effect on drinks/day, % days drinking, reduced % HDD (Knapp et al. 2015)	Decreased drinking in WHP rats (Zalewska-Kaszubska et al. 2011) Increased DID, decreased IA drinking in rats (Fish et al. 2014) Increased drinking P rats (Bell unpublished)
Galantamine	Cholinesterase inhibitor	No significant effect time to severe relapse (Mann et al. 2006)	Reduced drinking in AA rats (Doetkotte et al. 2005)

[a]Italic text indicates multisite trial

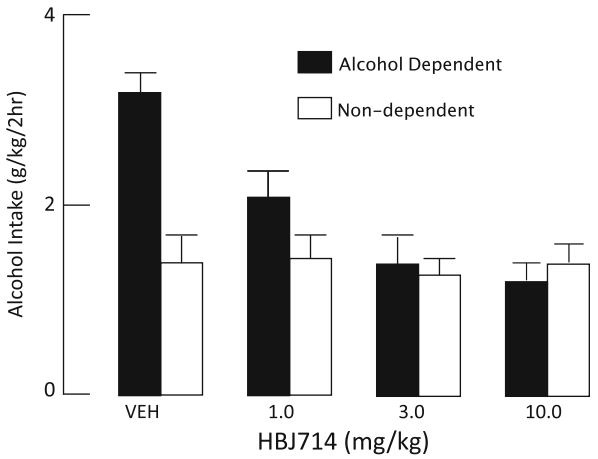

Fig. 1 HBJ714 reduces elevated alcohol drinking in dependent rats but is ineffective in nondependent rats. We conclude that HBJ174 reduces drinking by affecting pharmacological targets contributing to elevated drinking in the model. We infer that the same targets contributed to increased drinking in AUD patients and predict that HBJ714 will decrease alcohol drinking (and other AUD symptoms) in patients by engaging the target

negative outcome (e.g., model insensitivity, lack of target engagement) may never be rigorously pursued.

Preclinical medication efficacy tests should have distinct responses to clinically ineffective drugs. Some clinically ineffective medications reduce drinking in rodents (see Table 1, bromocriptine, buspirone, SSRIs), yet distinct effects are found for other clinically ineffective drugs. For example, a 15-day treatment with the SSRI fluoxetine produced long-lasting increases in alcohol drinking following periods of alcohol deprivation (Alén et al. 2013) which may correspond to increased relapse and drinking reported in some AUD patients after SSRI treatment (Chick et al. 2004; Dundon et al. 2004). Similarly, levetiracetam has been shown to increase drinking in mice (Fish et al. 2014), in rats (Bell unpublished data), and in non-treatment-seeking alcohol abusers (Mitchell et al. 2012a). In the future, identifying reliable and distinct preclinical test responses for ineffective AUD medications will temper indiscriminately positive preclinical test results when testing novel medications.

2 Preclinical Medication Evaluation: Two Concepts

Two complementary concepts arise when considering preclinical AUD medication evaluations. The first, introduced above, is the need for sensitive, reliable, predictive efficacy screens. The second concerns external validity in the form of parallel causal relationship structures among variables in the model and variables in the clinical domain.

2.1 Predictive Screening

Predictive screens require reference compounds of known clinical efficacy, standardized protocols, and blind, unbiased testing conditions. Some medications appearing in Table 1 may be suitable as reference standards. They are mechanistically diverse and the range is likely to expand. Whether a predictive efficacy screen can be constructed from them is unknown. The exercise has yet to be attempted.

Preclinical efficacy screens need not have obvious mechanistic relevance to AD to be predictive; however, the mechanistic range of reference compounds will determine the screen's scope. For example, screens based on only one positive (e.g., naltrexone) and one negative reference (e.g., fluoxetine) would discriminate opiate antagonists, SSRIs, and nothing else. Screens derived from a mechanistically diverse reference battery potentially discriminate a broader range of drugs through common, but unknown, mechanisms as exemplified by the DRL-72-s reinforcement schedule sensitive to mechanistically diverse classes of antidepressant drugs (O'Donnell et al. 2005). The mechanistic basis for the screen's predictive validity remains unknown.

Screening requires standardized protocols. Standardization eliminates the methodological variability found in the published literature, although it may not remove the smaller influences of unspecified variables over time and across laboratories (see Crabbe et al. 1999). Some argue (e.g., Richter et al. 2009) that standardization constrains external validity – i.e., the standardized methods sample too narrow a phenomenological space to be broadly generalizable. Nevertheless, "hits" identified by efficacy screens can be subjected to further tests to evaluate their robustness. As with clinical studies, blinding investigators to the compound's identity, mechanism of action, and test conditions is necessary to reduce testing biases.

Given the complexity of AUD and the pharmacological diversity of effective medications, a universal predictive screen may never emerge. For example, the recently developed HDID mouse line is not sensitive to naltrexone, but reduces alcohol intake following acamprosate and baclofen administration (Crabbe et al. 2017), whereas alcohol drinking in the alcohol-preferring msP rat is sensitive to naltrexone and acamprosate treatment, but not to varenicline (Scuppa et al. 2015). Therefore, it is possible that screens will consist of tests relevant to specific clinical indications, distinct patient subtypes, or specific therapeutic mechanisms. Positive effects on any of them would be considered further (Egli 2005). The challenge remains to construct the screens.

2.2 Mechanistic Modeling

The first acamprosate test in a preclinical alcohol drinking model used a subset of rats (24%) drinking alcohol solutions as 60% or more of their total fluid intake (Boismare et al. 1984). Following this, Jacques Le Magnen et al. (1987) showed that acamprosate reduced alcohol intake by rats rendered alcohol dependent, but had little effect in nondependent rats, thus, following the principle that preclinical

paradigms modeling AD would be sensitive to therapeutically effective drugs. Subsequently, investigators developed specialized voluntary drinking paradigms to engender high alcohol intake (>5 g/kg) and intoxicating blood alcohol concentrations (80–100 mg/dL or higher). Models include genetic (e.g., selectively bred P rats, msP rats, HDID mice), scheduled access (e.g., drinking in the dark [DID], alcohol deprivation [ADE]), and chronic exposure/extended access paradigms (e.g., chronic intermittent ethanol [CIE] exposure).

Pathologically relevant AD models led to the discovery of gene networks and brain mechanisms for consideration as AUD pharmacotherapy targets (e.g., Kimpel et al. 2007; Osterndorff-Kahanek et al. 2015). Elevated drinking in many AD models is selectively sensitive to therapeutically effective drugs relative to drinking in models more relevant to social drinking (see Meinhardt and Sommer 2015; Sprow and Thiele 2012; Tunstall et al. 2017). Metaphorically, excessive drinking models capture portions of the neurobiological "landscape" of alcohol dependence. Attenuating heightened alcohol intake with a test drug suggests that neurobiological targets relevant to AD are changed in a way that may ameliorate AUD in humans (see Fig. 1).

Testing drugs in animal AD models to draw inferences about clinical efficacy invokes the analogical argument, a formal statement that known similarities between a model and its target domain (i.e., the modeled clinical phenomena) permits us to conclude that additional features observed in the model will also be observed in the target domain (Bartha 2010). In Fig. 1, the implied analogical argument is of the form: HBJ714 decreases alcohol drinking in the model; therefore, it will reduce drinking in AUD patients.

Material or physical similarities (often called "face validity") between a model and its target are often discussed (Koob and Zimmer 2012). Less appreciated are similarities between causal relationships among variables within the model and causal relationships among corresponding variables in the model's target domain (see Hempel 1965; Ruse 1973). Figure 2 depicts the causal relationship structure of three categories of excessive drinking animal models (left) and the corresponding relationship among analogous clinical variables (right). At a broad level, there are parallels in causal relationship structures in the models and in aspects of clinical AUD.

Parallel causal relations are essential to the analogical argument (Hesse 1966; Bartha 2010), but the analogical argument also requires some degree of material similarity between corresponding variables (Hesse 1966, p. 69). In this context, the *initial analogy* – where a variable of strong interest is created in the model system – links the model and its target (Overmier and Patterson 1988). The initial analogy is often at the level of pathology such as in animal models of excessive alcohol drinking. Once the initial analogy is established, causally related variables are studied in the model system. As illustrated in Figs. 1 and 2, investigational drug HBJ714 – at a range of doses – decreases alcohol drinking in alcohol-dependent animals exposed to chronic intermittent ethanol vapors, but not drinking in an alcohol naïve group. From this, we infer that neurobiological systems dysregulated by the CIE procedure are targeted by HBJ714 to reduce drinking and that these same

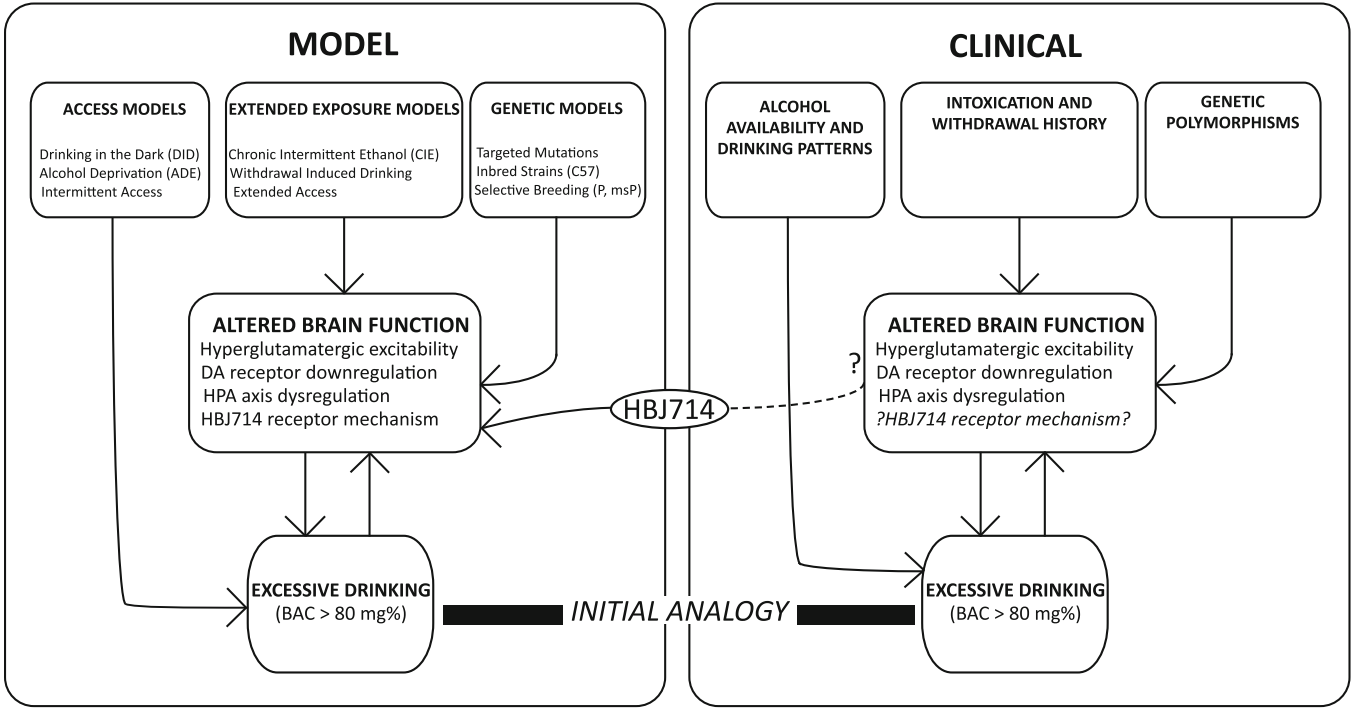

Fig. 2 Summary of the causal structure of excessive alcohol drinking models (left) and the corresponding clinical phenomena they represent (right). Arrows indicate causal relationships. Excessive drinking is the model's initial analogy (Overmier and Patterson 1988) which, in this example, corresponds to excessive drinking found in clinical AUD. According to Hesse (1966), material similarities between variables causally close to the initial analogy are essential; dissimilarities between causally distal conditions are usually tolerated. In the models, it is acknowledged that humans are not selectively bred and do not inhale alcohol vapors, but we expect brain states causally related to increased drinking in the models to be like those in AUD patients (e.g., glutamatergic excitability). As illustrated, we observe that HBJ714 decreases drinking in the AUD model and infer that HBJ714 will ameliorate excessive alcohol drinking clinically through similar brain mechanisms

neurobiological systems are likely to be targeted by HBJ714 in AUD patients to ameliorate clinical symptoms of AUD.

Do inferences drawn from preclinical AD models discriminate positive from negative RCT outcomes? As shown in Table 1 and discussed previously (Egli 2005; Koob et al. 2009; Yardley and Ray 2017), preclinical AD models are sensitive to clinically effective medications. Table 1 also suggests that, perhaps because there is no further interest, many clinically ineffective drugs have not been systematically examined in AD models and compared to effective medications. Because they do not model all the relevant variables present in RCTs, it may not be appropriate to expect preclinical AD models to completely discriminate effective from ineffective AUD medications. In the concluding sections, I will discuss strategies for translating preclinical to clinical studies. For now, it is important to appreciate that gaps between preclinical AD models and RCTs more likely reflect ignorance regarding the full spectrum of variables operating in the clinical setting than general deficiencies in modeling approaches (see Egli et al. 2016).

2.3 Inferences and the Exploratory-Confirmatory Continuum

Test outcomes must be interpreted in the context of prior knowledge. According to Bayes Theorem, estimating the probability that HBJ714 is clinically effective for AUD after a positive preclinical test outcome requires that the probability that a drug will be clinically effective given a positive test outcome (true positive rate), the test's false positive rate, and the probability that HBJ714 is clinically effective prior to the test all be considered.

Bayes theorem, stated mathematically, is:

$$p(A|X) = \frac{p(X|A)p(A)}{p(X|A)pA + p(X|\text{not } A)\, p(\text{not } A)}$$

where $p(A|X)$ is the probability the drug is clinically effective (A) given a positive test (X), $p(X|A)$ is the probability of a positive test (X) given that drug is clinically effective (A), $p(A)$ is the probability the drug is clinically effective, (not A) is the probability the drug is clinically ineffective, and $p(X|\text{not } A)$ is the probability of a positive test (X) given that the drug is clinically ineffective (not A).

Prior probability is informed not only by previous test outcomes but also relevant basic mechanism studies, preclinical and clinical efficacy evaluations of closely related drugs, and the overall success rate for AUD drugs, among other things. For instance, if HBJ714 is a novel μ-opioid receptor antagonist, the pretest probability that HBJ714 is clinically effective might be reasonably estimated to be 0.67 based on the success of naltrexone and research findings on opioid mechanisms of alcohol drinking. Let us also say that our initial preclinical test has performed somewhat better than a coin toss with a true positive rate of 0.67 and false-positive estimate of 0.47. In this scenario, the chance that HBJ714 will be clinically effective given a positive outcome in the preclinical test is reasonably high, about 0.74. (Note: we are

not considering possible adverse side effects and other significant drug development milestones in this example). Taking the first test into account, a subsequent positive result in a test with the same true positive and false-positive rates yields a probability estimate of 0.80. Yet another positive result using the same quality test yields 0.85, and another would only increase the probability estimate to 0.89. Assigning quantitative probability estimates is not realistic at present, and the numbers used in the example are not meant to be definitive or valid. Nevertheless, the example illustrates that successive testing gives diminishing returns. For a drug mechanistically very similar to successful medications, one or two positive preclinical efficacy test results may be sufficient.

Consider a second scenario where HBJ714 is a novel drug for which there is no prior mechanistic information with respect to AUD. The prior probability, $p(A)$, would be low. (Most drugs are not clinically successful). Let us be optimistic and set $p(A)$ equal to 0.10. In this case, after an initial successful test, the estimated chance that HBJ714 is clinically effective for AUD is only 0.14. Several successive positive test results (assuming the tests are similar in their true-/false-positive rates) would be required before we would have sufficient confidence in the drug's potential clinical efficacy. Of course, any negative test results – with their own set of probability estimates – would also influence the estimate.

The above examples suggest that the most basic research and efficacy evaluation practices are consistent with Bayes theorem. Negative test outcomes are tolerated when prior evidence is strong, whereas a positive test outcome in the face of overwhelmingly discouraging evidence has little influence unless previous findings have been adequately explained. Furthermore, tests and models are weighted according to perceived true- and false-positive rates, although they are seldom calculated. On this point, the Bayesian importance of a model's past predictive performance is complemented by a Popperian emphasis on risky predictions (see, e.g., Popper 1959). Models that exclusively generate observations easily predicted without the model (i.e., when $p(A)$ is high) are of lesser value than those revealing completely unexpected, confirmed information (i.e., when $p(A)$ is low). In simpler words, a system allowing us to successfully bet on long-shots that payoff is more valuable than one only capable of selecting favorites to win.

Research is performed in exploratory or confirmatory modes. Each mode has defining characteristics falling along a continuum rather than the dichotomy implied by some (Jaeger and Halliday 1998; Wagenmakers et al. 2012; Kimmelman et al. 2014). Early preclinical screens and mechanistic evaluations are exploratory. As less costly efforts, they generate hypotheses. In the pursuit of discovery, efforts are made to reduce false negatives and deviations from protocol standards are tolerated as tests are refined and new information is acquired. Successful exploratory research justifies pursuing riskier, resource-intensive confirmatory studies such as RCTs. Hypothesis supporting confirmatory research demands rigorous, outcome-neutral testing conditions, reduced risk of false-positive outcomes, and a willingness to accept the outcome – positive or negative – as definitive. Exploratory and confirmatory research each has its value if confirmatory conclusions are not drawn from exploratory data (Wagenmakers et al. 2012). Given that preclinical tests are models

of clinical phenomena, they are never fully confirmatory. Negative exploratory research findings – especially in the context of strong prior evidence – might call for a return the "drawing board" to refine hypotheses and measures rather than abandonment. Depending on the potential epistemological or therapeutic payoff, time, and financial and material resources, negative confirmatory research findings (such as a well-designed RCT) may occasionally retreat toward the exploratory mode, rejection and abandonment of the hypothesis, but seldom further confirmatory research efforts.

3 Discoveries

In this section, I discuss recent advances in preclinical AUD pharmacotherapy. For each target, there are clinically approved medications for conditions other than AUD. The selection is not comprehensive, but provides perspectives on preclinical AD models in the context of AUD pharmacotherapy development.

3.1 Glucagon-Like Peptide-1 (GLP-1) Receptor

GLP-1 is secreted from endocrine L cells in the intestine in response to nutrients. It is also produced and released in the brain, specifically, the nucleus of the solitary tract (NST) which projects throughout the CNS including the BNST and CeA – areas involved in the development of AD (Gu et al. 2013). GLP-1 regulates appetite and eating, gastric emptying, and glucose metabolism. The GLP-1 receptor, a Gs coupled GPCR, is expressed throughout the CNS including the brainstem, hypothalamic nuclei, VTA, NAc, and CeA – brain regions associated with addiction (Pratley and Gilbert 2008). GLP-1 itself is not used therapeutically because it degrades rapidly. Analogs with longer half-lives have been discovered or synthesized. Notably, exenatide – a synthetic analog of the exendin-4 hormone found in Gila monster saliva – is marketed for the treatment of type 2 diabetes.

Egecioglu et al. (2013) showed that exendin-4 attenuated alcohol-induced locomotor stimulation and NAc dopamine release, reduced alcohol CPP in mice, and decreased alcohol drinking and SA in rats after months of intermittent alcohol access. These effects were replicated with GLP-1 (Shirazi et al. 2013) and the GLP-1 receptor agonist liraglutide (Vallöf et al. 2016).

Shirazi et al. (2013) confirmed the involvement of endogenous GLP-1 in regulating alcohol intake by demonstrating that GLP-1 receptor blockade increased alcohol intake. CNS mediation was demonstrated when GLP-1 infusion into the VTA was shown to reduce alcohol intake (Shirazi et al. 2013). Sørensen et al. (2016) found that exendin-4 attenuated intravenous ethanol self-administration in mice, confirming that GLP-1 receptor activation reduced alcohol reinforcement independent of its effects on ingestive physiology.

Support for the GLP-1 receptor as a therapeutic target for AUD comes from observations in AD-relevant models. Shirazi et al. (2013) observed that reduced

alcohol intake following exendin-4 or GLP-1 administration was confined to the top 30% alcohol drinkers and was not detected in low-alcohol-consuming rats (bottom 30%). A similar observation was reported with liraglutide (Vallöf et al. 2016). Acute liraglutide administration prevented the ADE in rats (Vallöf et al. 2016) and daily exendin-4 administration blocked the ADE in mice Thomsen et al. (2017). Liraglutide also diminished alcohol SA in alcohol-preferring sP rats (Vallöf et al. 2016).

In a study performed prior to Egecioglu et al. (2013), but published later (Suchankova et al. 2015), effects of the GLP-1 agonist AC3174 were examined on drinking by alcohol-dependent mice and nondependent mice. In nondependent mice, AC3174 did not affect voluntary alcohol consumption, nor was it effective in reducing elevated alcohol drinking in dependent mice when first administered. When tested over additional alcohol-vapor-exposure cycles, AC3174 decreased escalated drinking. The effect, once established, was relatively long lasting. Only weeks later did alcohol consumption in dependent mice return to pretreatment alcohol intake levels. The time-dependent effects suggest that AC3174 requires repeated administration to be effective, that repeated alcohol-exposure cycles are required to sensitize GLP-1 relevant neurophysiology or both.

A recent comprehensive study suggests that GLP-1 neurons regulate nicotine intake in a manner analogous to their role in meal patterning (Tuesta et al. 2017). Specifically, nicotine stimulates GLP-1 neurons in the NTS to enhance the activity of excitatory habenular inputs to the interpeduncular nucleus (IPN). GLP-1 signaling in the IPN, in turn, blocks nicotine reward and promotes nicotine avoidance. Whether such a mechanism acts in concert with GLP-1 signaling in the VTA (Shirazi et al. 2013) to reduce alcohol intake remains to be studied.

Preclinical support for the GLP-1 receptor as an AUD therapeutic target is consistent with evidence that GLP-1 receptor variants are associated with AUD in humans (Suchankova et al. 2015). Preliminary functional validation was obtained retrospectively from two studies in which the GLP1R 168Ser allele was associated with increased alcohol SA and higher BOLD signal at the globus pallidus following rewarding feedback. It is notable that a clinical trial has been initiated in Denmark to investigate the effects of exenatide (Bydureon) on alcohol intake in AUD patients (ClinicalTrials.gov Identifier: NCT03232112). A remaining consideration for future clinical testing is that, at higher doses, GLP-1 agonists are anxiogenic in rodents through action in the amygdala (Möller et al. 2002; Anderberg et al. 2016). It remains unknown whether GLP-agonists affect anxiety during alcohol abstinence.

3.2 Phosphodiesterase (PDE) Inhibition

Neuroimmune/neuroinflammatory pathway regulation of alcohol drinking is discussed elsewhere in this volume (Coleman and Crews 2018; Roberto et al. 2018) and in recent reviews (Robinson et al. 2014; Crews et al. 2017). I discuss here preclinical studies supporting PDE inhibition as a pharmacotherapeutic strategy for alcohol dependence.

A negative relationship between brain cAMP signaling and alcohol consumption is well documented (e.g., Misra and Pandey 2006; Pandey et al. 2005; Logrip 2015) and suggests that pharmacologically enhancing cAMP signaling in relevant brain sites may reverse excessive alcohol drinking. PDE enzymes catalyze hydrolysis of cAMP and cGMP to inactive forms. The PDE4 enzyme family selectively inactivates cAMP suggesting that PDE4 inhibitors might be a novel therapeutic strategy to reduce excessive alcohol consumption (Wen et al. 2015).

Higher PDE4A gene expression levels were found in the NAc shell of P vs NP rats (Franklin et al. 2015). In addition, selective PDE4 inhibitors were found to decrease drinking by mice (Hu et al. 2011; Liu et al. 2017), alcohol SA and drinking by Fawn-hooded rats (Wen et al. 2012), and 2-h access drinking by HAD1 and P rats (Franklin et al. 2015). The selective PDE4 inhibitor rolipram also reduced anxiety- and depression-like behaviors in early and extended alcohol withdrawal (Gong et al. 2017). Blednov et al. (2014) compared PDE inhibitors with different subtype selectivity for their ability to reduce alcohol drinking and preference in C57BL/6J mice. Four PDE4 inhibitors – rolipram, mesopram, piclamilast, and CDP840 – decreased alcohol drinking and preference, whereas PDE1,3,5 and nonspecific inhibitors did not.

In addition to PDE4, PDE10 – a dual-specificity cAMP/cGMP inhibiting enzyme – has been implicated in AD. Gene network analysis consistently supports an association between brain pde10a gene expression and alcohol drinking in rodents (Mulligan et al. 2006; Wolstenholme et al. 2011; Osterndorff-Kahanek et al. 2015). Pde10a mRNA expression in the prelimbic subdivision of the mPFC and BLA was positively associated with relapse-like SA in rats with a history of stress exposure (Logrip and Zorrilla 2012) and was also elevated during both acute and prolonged abstinence from CIE exposure (Logrip and Zorrilla 2014). The selective PDE10A inhibitor TP-10 decreased alcohol SA in rats with a stress history, alcohol-dependent rats, sP rats, and stress-naïve, nondependent rats (Logrip et al. 2014). TP-10 also reduced saccharin SA suggesting that PDE10A may have a nonselective effect on motivated behavior.

Despite the availability of specific PDE4 and PDE10a inhibitors for human use, no clinical investigation on their effects on human alcohol drinking has yet been performed. Nevertheless, the nonspecific PDE3,4,10,11 inhibitor ibudilast – an anti-inflammatory drug used to treat asthma – decreased alcohol drinking in P and HAD1 rats and selectively decreased drinking in alcohol-dependent mice relative to nondependent mice (Bell et al. 2015). An exploratory human laboratory study tested ibudilast effects on subjective response to alcohol administration and to cue- and stress-induced changes in alcohol craving and mood in non-treatment-seeking AUD subjects (Ray et al. 2017a). Ibudilast did not significantly affect subjective response to alcohol, but improved mood responses to stress and alcohol cue exposure and decreased alcohol craving. Post hoc analysis revealed that ibudilast decreased alcohol's stimulant and mood-altering effects in subjects with higher (subclinical) depressive symptoms, an effect that may be relevant to antidepressant actions of PDE4 inhibitors (Fleischhacker et al. 1992). Given negative mood associated with protracted abstinence and relapse (Koob 2015), selective reduction of post-

dependent drinking (Bell et al. 2015), and decreased post-dependent anxiety- and depression-like behaviors following rolipram treatment (Gong et al. 2017), further studies on PDE4 inhibition and its effects on mood and craving during the post-dependent state are warranted.

Ibudilast's inhibitory effects on PDEs are greatest for the 4-type enzyme. Its effects on alcohol drinking might occur through other mechanisms, however, although it is unlikely that TLR4 antagonism is one of them (Harris et al. 2017). Additional studies should clarify PDE or other mechanisms through which ibudilast affects alcohol drinking and other AD-relevant measures. In addition, clinically approved selective PDE4 inhibitors are available for testing in AUD subjects; however, they are associated with undesirable side effects such as nausea and emesis – actions attributed to the PDE4D subtype. PDE4B subtype-specific drugs under development may eliminate the undesirable side effects while retaining action in brain regions associated with affect and motivation (Cherry and Davis 1999).

3.3 Glucocorticoid Receptor (GR)

The glucocorticoid receptor (GR) is a widely expressed transcription factor mediating diverse physiological responses to glucocorticoids (Oakley and Cidlowski 2013). It is surprising that the GR has only recently been considered as a target for AUD pharmacotherapy given the well-known relationship between stress physiology and AUD. Alcohol intoxication and withdrawal activate the hypothalamus-pituitary-adrenal (HPA) axis to elevate circulating glucocorticoids (cortisol [CORT] in primates, corticosterone [CORT] in rodents) in rodents (Tabakoff et al. 1978; Rasmussen et al. 2000), monkeys (Schwandt et al. 2011), and humans (Adinoff et al. 1998). CORT levels remain elevated in specific brain regions months after alcohol withdrawal (Little et al. 2008) to engender a blunted HPA axis response characteristic of long-term heavy alcohol exposure (Lee and Rivier 1997; Richardson et al. 2008; Helms et al. 2012) and protracted alcohol abstinence (Adinoff et al. 1990; Wand and Dobs 1991).

Alcohol intake is increased by glucocorticoids (Fahlke et al. 1994; Fahlke and Eriksson 2000). Whether these effects are mediated through GR interactions with brain DA reward circuitry as proposed for psychomotor stimulants (Piazza and Le Moal 1997) is unknown. Under nondependent conditions, the GR antagonist mifepristone (RU486) decreased limited access alcohol drinking in rats (Koenig and Olive 2004) and blocked alcohol CPP acquisition (Rotter et al. 2012) suggesting that GR mediates alcohol reward and nondependent alcohol drinking.

Prolonged HPA axis activation and CORT release caused by frequent alcohol intoxication and withdrawal escalates alcohol intake through two distinct GR mechanisms (Edwards et al. 2015; Tunstall et al. 2017). CORT activation of the GR in the PVN inhibits CRF release which, in turn, diminishes HPA activation. With prolonged drinking, this leads to the dampened HPA function and subsequent binge drinking to temporarily restore HPA axis tone (Blaine and Sinha 2017). In addition, GR activation in the CeA by CORT stimulates CRF release and increases

GR expression (Vendruscolo et al. 2012) giving rise to negative affect and increased alcohol drinking through negative reinforcement mechanisms (Edwards and Koob 2010). To that end, mifepristone prevented escalation of alcohol drinking in rats exposed to chronic intermittent alcohol vapors (Vendruscolo et al. 2012) and mifepristone, and the selective GR antagonist CORT113176 selectively reduced alcohol drinking in dependent rats (Vendruscolo et al. 2015). Mifepristone administered into the CeA also blocked yohimbine-induced reinstatement of extinguished alcohol SA (Simms et al. 2012).

Glucocorticoid-induced neurotoxicity arising from long-term alcohol intoxication also contributes to cortically mediated cognitive dysfunction that may impair regulation of alcohol intake (Rose et al. 2010; Lu and Richardson 2014; Blaine and Sinha 2017; Pahng et al. 2017). The GR is expressed in the PFC and coordinates stress response in multiple brain regions. Chronic ethanol intoxication and withdrawal alters GR signaling in the mPFC (Somkuwar et al. 2017). GR antagonism during alcohol withdrawal in rats was shown to ameliorate memory impairments in mice (Jacquot et al. 2008) suggesting that, with the PVN and CeA, GRs in the PFC also serve as a pharmacotherapeutic target for AUD.

With strong preclinical support, mifepristone was tested in non-treatment-seeking AUD subjects over a 1-week period (Vendruscolo et al. 2015) and found to reduce alcohol cue-induced craving in the laboratory and decrease alcohol consumption during the treatment phase and 1-week posttreatment. Additionally, results of a clinical proof-of-concept study examining a 14-day mifepristone treatment on cognitive function and mood after drinking cessation awaits publication (Donoghue et al. 2016). The strong preclinical support and encouraging clinical results justify further clinical studies with mifepristone or novel selective GR antagonists.

3.4 Nociceptin (NOP) Receptor

Nociceptin/orphinin FQ (N/OFQ) is the endogenous ligand for the nociceptin receptor (NOP) – a G-protein-coupled receptor sharing significant homology with classical opioid receptors, yet having little to no affinity opioid peptides or morphine-like drugs (Meunier et al. 1995; Reinscheid et al. 1995). Although of interest for pain reduction and feeding effects, N/OFQ attenuates stress-like responses and has a broad anxiolytic profile. Relevant to AUD, the NOP receptor expressed brain regions associated with alcohol intake and AD including the BNST, CeA, PFC, VTA, NAc, and LC (Witkin et al. 2014).

Preclinical evidence for NOP receptor involvement in AUD has been reviewed elsewhere (e.g., Murphy 2010; Witkin et al. 2014). The studies showed that nocioceptin and brain-penetrant NOP receptor agonists reduced alcohol drinking and SA (Ciccocioppo et al. 1999, 2014) and alcohol CPP (Kuzmin et al. 2003). They also decreased alcohol SA in alcohol-dependent rats at doses that were ineffective in nondependent rats (de Guglielmo et al. 2015), blocked ADE development (Kuzmin et al. 2007), and blocked footshock (Martin-Fardon et al. 2000) and cue reinstatement of extinguished alcohol SA (Ciccocioppo et al. 2004). In the rat, ethanol

significantly augments CeA GABA release, whereas N/OFQ diminishes it, an effect that is significantly stronger in alcohol-dependent rats (Roberto and Siggins 2006). These observations, combined with reduced pronociceptin and NOP receptor expression observed in postmortem brain tissue of human alcoholics (Kuzmin et al. 2009), support the hypothesis that endogenous N/OFQ functions to limit alcohol intake. Restoring diminished brain NOP receptor-system function through NOP receptor agonist administration, therefore, seemed to be a valid therapeutic strategy for AUD.

Contradictory observations appeared early in a report that N/OFQ administered continuously via an indwelling minipump elevated already substantial alcohol drinking by msP rats (Cifani et al. 2006). Recently, rats carrying a deletion of the NOP receptor self-administered less alcohol and reached lower progressive-ratio breakpoints than wild-type rats (Kallupi et al. 2017). These studies suggested a positive rather than negative relationship between nociceptin activity and alcohol intake. Accordingly, NOP receptor antagonists rather than agonists would be the desired AUD pharmacotherapy.

Earlier, when selective NOP receptor antagonists were used as experimental tools to verify NOP receptor activity in agonist studies, they had no significant effect on alcohol intake (Ciccocioppo et al. 2002, 2007) yet, in the latter study – which did not report the p-value – there appeared to be reduced drinking at the middle dose (see Ciccocioppo et al. 2007, Fig. 3). In preparation for clinical testing, Rorick-Kehn et al. (2016) found that LY2940094, a brain-penetrant, selective NOP receptor antagonist, significantly reduced home cage alcohol drinking and operant alcohol SA breakpoints in female P rats and male msP rats. LY2940094 also blocked yohimbine-induced reinstatement of extinguished alcohol SA by msP rats and blocked alcohol-stimulated DA release in the NAc. These preclinical observations served as the basis for an 8-week, double-blind, placebo-controlled, proof-of-concept study in 88 AUD patients (Post et al. 2016). Although the primary outcome measure, drinks per day, did not differ significantly between the treatment groups, LY2940094 significantly reduced % monthly heavy drinking days and increased % abstinent days per month.

The encouraging clinical efficacy for the NOP receptor antagonist LY2940094 raises questions regarding approximately 15 years of preclinical studies showing efficacy for NOP receptor agonists. Rorick-Kehn et al. (2016) hypothesized that chronic NOP receptor agonist administration downregulates the N/OFQ system through NOP receptor desensitization and internalization. Reduced NOP receptor expression at the plasma membrane available to bind N/OFQ results in a functional receptor blockade. Agonist effects reported previously may be related to reduced NOP receptor availability and diminished endogenous signaling after sub-chronic agonist administration. The hypothesis is consistent with reports that attenuating alcohol drinking and SA required repeated NOP receptor agonist administration (Ciccocioppo et al. 1999, 2014; Economidou et al. 2006, 2008). Agonist-induced NOP receptor desensitization and internalization has been examined in vitro (Dautzenberg et al. 2001), but not in parallel with alcohol drinking.

Until we understand NOP receptor dynamics in response to repeated agonist and antagonist administration, concerns remain about detrimental effects that could worsen AUD. Like their effects on alcohol drinking, NOP receptor agonists (Vitale et al. 2006; Lu et al. 2011) as well as antagonists (Duzzioni et al. 2011) have anxiolytic effects. NOP agonists decrease anxiety-like behavior in acute and protracted alcohol withdrawal (Economidou et al. 2011) possibly through functional CRF antagonism in the BNST (Rodi et al. 2008) and CeA (Cruz et al. 2012). If NOP receptor antagonism – functional or direct – is required to decrease alcohol drinking and related AD measures, concerns remain regarding potential anxiogenic effects, particularly during abstinence, after extended NOP receptor agonist or antagonist administration. Preclinically, N/OFQ attenuated elevated anxiety-like behavior in ethanol-dependent rats 1-week post-withdrawal but was anxiogenic 3-week post-withdrawal (Aujla et al. 2013). Expanding the canvas, NOP antagonists also have antidepressant actions which are reversed by NOP agonists (Gavioli and Calò 2013). For the NOP receptor to be a serious therapeutic AUD candidate, further preclinical studies are needed to evaluate anxiolytic and antidepressant responses in parallel with alcohol-related measures. Ligands over a range of intrinsic activities may also need to be considered. Once resolved, the availability of PET ligands for the NOP receptor will support translation to human subjects (Narendran et al. 2017).

3.5 Corticotrophin-Releasing Factor (CRF) Receptor 1

CRF's involvement in alcohol dependence has been reviewed extensively (see Zorrilla et al. 2014; Phillips et al. 2015; Quadros et al. 2016; Schreiber and Gilpin 2018, this volume). Briefly, extrahypothalamic CRF1 receptors in the CeA (Roberto et al. 2010), MRN (Lê et al. 2013), DRN (Quadros et al. 2014), mPFC (George et al. 2012; Gondré-Lewis et al. 2016), VTA (Hwa et al. 2013; Sparta et al. 2013), and BNST (Pleil et al. 2015) mediate excessive alcohol intake in CIE and scheduled access models, reinstatement associated with early and proximal stress, and alcohol heightened aggression.

The CRF1 receptor as an AUD therapeutic target is supported by preclinical peptide and non-peptide drug studies using a diverse range of alcohol drinking and AUD models. Approximately 75% of published CRF1 antagonist tests show positive effects (I reviewed 46 publications reporting 63 distinct test outcomes for 12 CRF1 antagonists). The 25% negative outcomes were largely confined to 24-h access or limited access drinking or to alcohol cue reinstatement in nondependent, non-stressed animals – that is, non-pathological social drinking models. In mice drinking to BACs >100 mg% (Correia et al. 2015), alcohol-preferring msP rats (Gehlert et al. 2007), stressed rats (Roltsch et al. 2014), alcohol-dependent rats (Chu et al. 2007), or rats undergoing alcohol withdrawal (Funk et al. 2006), CRF1 antagonists reduced alcohol intake, anxiety-like behaviors (Gehlert et al. 2007), and brain stimulation reward thresholds (Bruijnzeel et al. 2010). CRF1 antagonists also reduced yohimbine-induced (Marinelli et al. 2007) and footshock-induced (Liu and Weiss 2002) reinstatement of extinguished alcohol SA – a response that is

stronger in msP rats (Hansson et al. 2006) and in alcohol-dependent rats (Gehlert et al. 2007). CIE exposure – under conditions sufficient to increase alcohol drinking and engender anxiety-like behaviors – increases Crh and Crh1 mRNA and transcript expression in the amygdala (Sommer et al. 2008; Eisenhardt et al. 2015) and leads to CRF1 receptor-mediated enhancement of GABA transmission in the CeA (Roberto et al. 2010). Innate upregulation of Crhr1 transcript was found in several limbic brain areas of alcohol-preferring msP rats as was a genetic polymorphism of the Crhr1 promoter (Hansson et al. 2006).

Two recent negative clinical studies examining CRF1 antagonist effects on alcohol craving are remarkable considering the abundant preclinical evidence for CRF1R as therapeutic target for AUD. In the first (Kwako et al. 2015), nonsignificant effects were likely due to the antagonist's receptor binding kinetics. The second study (Schwandt et al. 2016) used verucerfont, a CRF1 antagonist having a more effective pharmacokinetic profile. Verucerfont administration decreased neuroendocrine responses to the Trier social stress test combined with alcohol cues and attenuated amygdala responses to fearful face images. Personalized auditory-guided imagery scripts, the Trier test, and alcohol cues also increased experimental measures of alcohol craving and negative emotionality, but these responses were not significantly altered by verucerfont administration.

The two clinical studies suggest that the therapeutic scope of selective CRF1 antagonists for AUD may be narrower than hoped (Spierling and Zorrilla 2017). Yet, as discussed by Spierling and Zorrilla (2017), the absence of an alcohol cue response is consistent with animal studies showing that CRF1 antagonists do not affect cue reinstatement of extinguished alcohol SA (Liu and Weiss 2002). At present, there is no reason to believe that the negative human data arise from unknown distinctions between the human and rodent CRF system. The absence of reliable brain-penetrant PET radioligands targeting the CRF1 receptor or human postmortem CRF1 receptor brain mapping studies diminishes our understanding of the human CRF1 system. Nevertheless, nonhuman primate studies show remarkable consistency with rodent data with respect to amygdala CRF mediation of anxious temperament (Kalin et al. 2016; and see Koob 2016 commentary).

Additional subject characteristics in Schwandt et al. may have influenced their results. Because of CRF1-antagonist safety concerns in males, subjects were females. In addition, they recruited subjects with high trait anxiety (Spielberger State-Trait Anxiety Inventor (STAI) score >39). The subjects, therefore, could be thought to have anxiety problems distinct from AUD or perhaps reflect Cloninger's type I alcoholism. According to the preclinical literature, which was performed exclusively in male subjects, chronic drug exposure engages adaptations in CRF circuits within the extended amygdala. Instead of a tonic, persistent negative mood state, the resulting negative affective state has been characterized as a "dynamic, active response to an acute stressor" (Koob and Zorrilla 2012) having features like affective pain responses (Egli et al. 2012). Trait anxiety as measured by the STAI may be distinct from the CRF-mediated negative affect component of AD. Hence, a CRF1 antagonist may not have been the appropriate medication for the Schwandt et al. subjects.

The exploratory human CRF1-antagonist studies, rather than definitively disconfirming the involvement of CRF in human AUD or conclusively ruling out CRF1R as a therapeutic target, raise several important questions regarding CRF1R pharmacology, as well as sex, species, and individual differences in CRF receptor-mediated physiology and behavior that will repay richly if they are pursued rather than abandoned.

4 Translation and Back Translation

I reviewed five medication targets for which clinically approved drugs showed efficacy in preclinical AD models. Invoking the analogical argument, the preclinical studies support the plausibility that (1) the targets contribute to human AUD and (2) pharmacologically engaging the targets will ameliorate excessive drinking and other AD symptoms. For ibudilast, mifepristone, CORT113176, and LY2940094, early clinical studies were also encouraging. As illustrated in Fig. 2, preclinical models, by design, do not account for every influential clinical variable. The influence of the modeled variables on clinical outcome variables, relative to those not modeled (e.g., expectancies, social influences), will determine the model's predictive relationship. The predictive relationship, therefore, will vary across individuals and settings. The next step, however, is to address translation from animals to humans.

4.1 Advancing Translational Studies

At a 1956 National Institute on Mental Health workshop devoted to evaluating psychiatric pharmacotherapies in animals, the eminent behavioral psychologist B. F. Skinner proposed that:

> one moves from the experimental analysis at the lower level to the human level, not by pointing out possible analogies, but by constructing an experimental situation in which the same kind of variables are manipulated and the same changes in behavior measured. (Skinner 1959)

Skinner's prescient statement anticipated the current interest in translational approaches to AUD medication evaluation (e.g., Mason and Higley 2013; Bartlett and Heilig 2013; Kwako et al. 2017). Behaviors associated with AUD pathology are complex and challenging to model in animals, however. Focusing on simpler biological and behavioral laboratory measures in animals and in humans allows investigators to compare drugs across species directly. While, at first, simplification narrows the model's scope, it promotes translation. Scope can be expanded through additional studies.

Endophenotype-focused translational approaches (Anderzhanova et al. 2017) capitalize on hypothesized neurophysiological substrates of behavior. Treatment-

responsive examples for AUD medication evaluation include alcohol-induced DA release, tonic central glutamate levels, network connectivity, and stress hormonal responses (Ray et al. 2009; Heilig et al. 2016; Schwandt et al. 2016). Translational endophenotypic measures are constrained by availability of safe, noninvasive in vivo tools to measure molecular, cellular, and circuitry function in humans. Measures also require causal relevance to clinical variables of interest. For example, verucerfont blocked HPA axis response in rats and humans confirming drug activity in both species (Schwandt et al. 2016), but it did not affect alcohol craving suggesting that HPA axis response may not be a causally relevant biomarker for alcohol craving.

Constraints in measuring human brain function may be circumvented by using surrogate behavioral tests with known brain mechanisms. For example, Kaye et al. (2017) proposed cue-induced startle potentiation as a translational behavioral probe to assess neuroadaptive changes in extrahypothalamic stress systems. Although the measure has only been validated in animals, it is readily applied to humans and rodents. Similarly, Cservenka et al. (2017a) developed an alcohol-specific prediction-error task in humans derived from preclinical studies. Identifying further behavioral assays dependent upon distinct brain mechanisms and with causal relevance to AUD pathology will provide important translational tools.

Translational studies also reveal discrepancies between animals and humans. Rather than translational failures, they are opportunities for discovery. For example, species-appropriate stressors have temporally distinct effects on progressive-ratio alcohol SA breakpoints depending on whether subjects are humans (McCaul et al. 2017) or mice (Norman et al. 2015). Experimentally identifying stressor or species variables contributing to the distinct responses will enable the development of translational paradigms for medication testing.

Once we have moved confidently from animals to humans, translational biochemical and behavioral measures can be incorporated into RCTs to evaluate their predictive relationship to trial outcomes. Thus, translational approaches may resolve ongoing discussions about the impact of variables distinguishing non-treatment-seeking subjects used in laboratory alcohol studies from treatment seeking subjects used in AUD pharmacotherapy RCTs (Ray et al. 2017b; Rohn et al. 2017). Circling back, translational measures predicting primary RCT outcomes can serve as preclinical screens, and to the extent they are causally related to other definitive AUD phenotypes, they can be included in novel mechanistic models.

4.2 Outcome Measures and Therapeutic Signals

There is no consensus about the most relevant clinical outcome measures (see Witkiewitz et al. 2017 and associated commentaries). The question arises, then, whether preclinical research can guide selection of clinical outcome measures and other RCT design aspects. Excessive drinking is an important link between preclinical models and clinical AUD, yet, the emphasis on excessive drinking does not imply that reducing active drinking is the most important clinical outcome. Instead, excessive drinking in preclinical models is best considered as a reliable behavioral

"readout" of altered brain functioning relevant to AUD (Egli et al. 2016). Demonstrating drug effects in pathologically relevant models that do not require the presence of alcohol (e.g., CPP, reinstatement) supports the medication's potential efficacy in abstinent patients. With few exceptions, however (e.g., Liu and Weiss 2002), pathologically relevant animal models tend to be universally (or unsystematically) responsive or nonresponsive to test medications regardless of whether drinking, cue effects, or stress effects are measured. Preclinical studies, therefore, are essential for detecting efficacy signals, but they are likely to be only minimally useful guides for preferring one RCT outcome measure over another.

An additional challenge is that patients meeting diagnostic criteria for AUD are diverse. They differ in the specific criteria leading to their diagnosis, and they can be subtyped according to additional psychological, physiological, and life history measures. As a result, they respond differently to AUD medications (Litten et al. 2015). By extension, a medication's therapeutic signal should be enhanced by recruiting subjects representing specific, more homogeneous, patient populations most likely to benefit from the medication (Addolorato et al. 2013). Although subjects can be classified according to a spectrum of variables such as age of onset or reinforcement-based phenotypes, genetic markers constitute the most reliably measured. To that end, gene variants modulating AUD medication effects have been identified through secondary analyses and, in some cases, validated in animal models (Heilig et al. 2011; Jones et al. 2015; Cservenka et al. 2017b). Nevertheless, prospective stratification by genotype has met with mixed success; differential naltrexone responses were not observed as a function of A118G mu-opioid receptor gene variant (Oslin et al. 2015; Schacht et al. 2017), whereas serotonin transporter allelic variants significantly influenced ondansetron treatment response in a randomly assigned prospective study (Johnson et al. 2011).

Linking genes to complex disease states such as AUD is not simple. Using gene variants as predictors of treatment response assumes that their influence remains constant throughout the course of a disease (Heilig and Leggio 2016). In addition, causal relationships across biological and behavioral levels of analysis are complex. The reductionist view is that variables at the micro level (e.g., genes and proteins) constrain those at the macro level (emotions, behavior), yet it has been demonstrated that causal relationships observed at the macro level often do not hold at the micro level (Hoel et al. 2013). By implication, immediate downstream effects of AUD disease genes can be altered by drug treatment while having minimal impact in the larger causal environment of AUD recovery. Indeed, it is well established that therapeutic effects of pharmacological treatment are strongly influenced by many variables such as the magnitude of placebo effects (Litten et al. 2013a), physician expectations (Spagnolo et al. 2015), and the presence of concurrent behavioral interventions (Anton et al. 2008b). Therefore, systematically apprising the impact of medication responses – genetically influenced or not – in the broader therapeutic milieu may constitute a greater translational challenge than showing homology between animals and humans.

The nascent field of computational psychiatry may offer solutions to these challenges (see Redish and Gordon 2016). Taking advantage of increased

computational power, emerging digital biometrics, and advanced neuroscience technologies, computational psychiatry seeks to integrate observations across biological and behavioral systems (from genes to behavior) over time through sophisticated modeling to identify "fault points" leading to disease and to suggest effective therapies. Rather than attempting to encompass the larger scope of the computational model, preclinical studies will be essential for discovering and confirming critical functional relationships within the model. The resulting research enterprise will be radically different from traditional approaches and come nearer to those in the advanced sciences. For example, rather than attempting to confirm significant differences between medication and placebo conditions (i.e., null hypothesis rejection), researchers will strive to demonstrate that recovery parameters induced by treatment do not deviate significantly from those predicted by the model (i.e., failure to reject the null hypothesis). When these goals are realized – perhaps decades from now – the question as to whether observations derived from individual subjects or a tightly defined cohort generalize to a broader diagnostic category, like the diagnostic category itself, becomes only a minor concern.

5 Conclusion: What Do Preclinical AD Models Tell Us?

Investigational drug HBJ714 is effective in a preclinical AD model. The plausibility that HBJ714 is an effective AD medication will be determined by (a) the model's history of discriminating clinically effective from ineffective medications, (b) its material similarity to a definitive AD phenotype, (c) parallels between the model's causal structure and causal influences in clinical AD, (d) the influence of modeled variables on clinical outcome measures relative to variables ignored by the model, and (e) evidence from previous studies. The preclinical test result, therefore, justifies further research in the form of additional preclinical studies, translational human laboratory studies, and appropriately designed confirmatory RCTs. Whether the same conclusions and decisions would occur in the absence of the model determines the model's value.

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Advances in Pharmacotherapy Development: Human Clinical Studies

Raye Z. Litten, Daniel E. Falk, Megan L. Ryan, Joanne Fertig, and Lorenzo Leggio

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Abstract

For more than 25 years, researchers have made advances in developing medications to treat alcohol use disorder (AUD), highlighted by the US Food and Drug Administration's (FDA's) approval of disulfiram, naltrexone (oral and long-acting), and acamprosate. These medications are also approved in Europe, where the European Medicines Agency (EMA) recently added a fourth medication, nalmefene, for AUD. Despite these advances, today's medications have a small effect size, showing efficacy for only a limited number of individuals with AUD. However, a host of new medications, which act on variety of pharmacologic targets, are in the pipeline and have been evaluated in numerous human studies. This article reviews the efficacy and safety of medications currently being tested in human trials and looks at ongoing efforts to identify candidate compounds in human studies. As mentioned in the *National Institute on Alcohol*

R.Z. Litten (✉) · D.E. Falk · M.L. Ryan · J. Fertig
Division of Medications Development, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA
e-mail: rlitten@mail.nih.gov

L. Leggio
Section of Clinical Psychoneuroendocrinology and Neuropsychopharmacology, National Institute on Alcohol Abuse and Alcoholism and National Institute on Drug Abuse, Bethesda, MD, USA

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2017_79

Abuse and Alcoholism's Strategic Plan 2017–2021 (https://www.niaaa.nih.gov/sites/default/files/StrategicPlan_NIAAA_optimized_2017-2020.pdf), medications development remains a high priority. By developing more effective and safe medications, and identifying those patients who will benefit the most from these treatments, we can provide clinicians with the tools they need to treat this devastating disorder, providing relief for patients and their families and markedly improving public health and safety.

Keywords
Alcohol use disorder · Human studies · Medications development · Pharmacotherapy

1 Introduction

In the United States, three medications have been approved by the US Food and Drug Administration (FDA) for the treatment of alcohol use disorder (AUD): disulfiram, naltrexone (oral and extended-release injectable), and acamprosate. Yet most people, less than 20%, seek treatment for AUD during their lifetime, and of these less than 4% receive FDA-approved medications (Grant et al. 2015; Litten 2016). Medications for AUD have been available to clinicians for decades but remain underutilized. In an effort to better inform clinicians about the benefits of medications for treating AUD, the Substance Abuse and Mental Health Services Administration (SAMHSA) and National Institute on Alcohol Abuse and Alcoholism (NIAAA) recently published practical guidelines for prescribing these FDA-approved medications in clinical practice (http://store.samhsa.gov/shin/content//SMA15-4907/SMA15-4907.pdf).

Raising awareness about the medications available and offering guidelines for prescribing them may help. Still, clinicians' reluctance to make full use of pharmacotherapies may be, in part, because the existing medications have such small effect sizes, on average, and show a wide range of efficacy across patients. Some of this variability is due to the significant heterogeneity of AUD itself. We simply do not know which type of patient is most likely to respond to each of these medications. Severity, duration, and family history of the disorder, co-occurring disorders, demographic characteristics, and genetic markers, among other factors, all can impact how a patient responds to medication. In other words, there is no silver bullet for the treatment of this complex disorder.

Because of this, in recent years, the search for medications to treat AUD has undergone a paradigm shift toward precision medicine. Medications development is now actively seeking to identify subgroups of patients who will benefit the most from specific compounds and have the fewest side effects. The goal is to provide clinicians with a full menu of medication options from which to choose. This is not an entirely new concept. Over two decades ago, NIAAA first explored the idea of "matching" specific treatments to specific types of patients with AUD in a series of groundbreaking studies conducted through the *Project Match* initiative (Project MATCH Research Group 1997). Since then, with advances in neuroscience and the underlying etiology of AUD, clinical trials have focused not only on whether a

medication works "on average" across a patient population but also if it works better in certain patient subgroups based on a purported mechanism of action.

Most encouraging is the diversity and number of targets being studied today, as well as the number of potential compounds currently in development. Preclinical work is summarized in another chapter of this book (see Egli, M). Here we detail the latest research on these medications, including their mechanism of action, efficacy, and safety in treating AUD in humans. We include medications that have exhibited efficacy in alcohol treatment clinical trials, medications that initially showed promise but failed in subsequent multisite clinical trials, as well as medications which are still in the early stages of evaluation in human studies (Table 1).

1.1 Medications Exhibiting Efficacy in Alcohol Treatment Clinical Trials

1.1.1 Nalmefene

Molecular and Functional Mechanisms of Action

Nalmefene is an opioid antagonist approved by the FDA to treat opioid overdose. Although structurally similar to naltrexone, nalmefene has several differences that may have advantages over naltrexone. It has a higher affinity for mu and kappa opioid receptors, a lower likelihood to induce liver toxicity, and higher bioavailability than naltrexone (Mann et al. 2016; Mason et al. 1999). In a three-group (nalmefene, naltrexone, and placebo) human laboratory study, nalmefene reduced alcohol-induced craving and stimulation compared with placebo. Similar results also were found for naltrexone (Drobes et al. 2004).

Results of Clinical Trials

In a single-site 12-week randomized clinical trial (RCT), Mason et al. (1999) first showed that alcohol-dependent patients who received either 20 mg or 80 mg of nalmefene per day were less likely to relapse to drinking or to drink heavily, compared with patients who were treated with placebo. Results were not significantly different between the 20- and 80-mg doses, with both nalmefene groups showing similar efficacy. In another 12-week multisite RCT in 270 alcohol-dependent patients, nalmefene, evaluated at three different doses (5, 20, and 40 mg per day), failed to show a difference in the number of heavy-drinking days (defined as five or more drinks per day for men and four or more drinks per day for women), alcohol craving, gamma-glutamyl transpeptidase (GGT), and carbohydrate-deficient transferrin (CDT) levels (GGT and CDT are biomarkers of alcohol consumption) (Anton et al. 2004). In contrast to the Anton et al. (2004) study, three recent large RCTs of nalmefene showed a positive effect on drinking outcome, which, subsequently, led to approval of this medication for treatment of AUD in Europe. The first 24-week RCT was conducted in 604 alcohol-dependent patients across 39 sites in Austria, Finland, Germany, and Sweden (Mann et al. 2013). Nalmefene was taken as

Table 1 Medications to treat alcohol use disorder

Medication (dose)	Site of action	FDA approval	Efficacy	Common side effects
Medications exhibiting efficacy in alcohol treatment clinical trials				
Nalmefene (18 mg/day as needed)	Opioid antagonist	Reversal of opioid overdose. Approved for alcohol dependence in Europe	Small effect in reducing drinking in three recent European trials	Nausea, vomiting, fatigue, insomnia, and dizziness
Varenicline (2 mg/day)	Partial α4β2 and full α7 nicotinic agonist	Smoking cessation	Recent multisite RCT showed varenicline reduced drinking in alcohol-dependent smokers and nonsmokers. Varenicline may be most effective in people with less severe AUD and among smokers who reduced their smoking	Nausea, abnormal dreams, and constipation. In rare instances, seizures may occur. In some individuals, varenicline may affect their ability to tolerate alcohol
Topiramate (100–300 mg/day)	Facilitates GABA activity, glutamate AMP and kainite antagonist, blocks L-type calcium channels, reduces voltage-dependent sodium channel activity, inhibits carbonic anhydrase	Partial and tonic-clonic seizures and migraines. Combination of topiramate and phentermine as adjunct for obesity	Several RCTs, including one multisite study, have demonstrated topiramate's efficacy in reducing alcohol consumption. Recent study indicated that genetic polymorphism in GRIK1 gene predicts a more favorable response to topiramate with fewer side effects	Dizziness, paresthesia, memory or concentration impairment, psychomotor slowing, nervousness, taste perversion, pruritus, and weight loss
Zonisamide (400–500 mg/day)	Enhances GABA activity, blocks voltage-sensitive sodium channels, blocks T-type calcium channels, inhibits carbonic anhydrase	Adjunct for partial seizure	In preclinical, human laboratory and open-label studies, zonisamide showed promise for treating AUD. This was confirmed by two single-site RCT trials, which showed zonisamide was effective in reducing alcohol consumption. Zonisamide appears to exhibit efficacy similar to topiramate	Side-effect profile is similar to topiramate, although somewhat less severe
Gabapentin (600–1,800 mg/day)	Appears to interact with voltage-gated calcium channels to indirectly modulate GABA activity	Adjunct for partial seizure, neuropathic pain, restless legs syndrome	Several single-site RCTs demonstrated gabapentin is effective in increasing abstinence and the number of no heavy-drinking days	Fatigue, insomnia, and headaches
Baclofen (30–80 mg/day)	$GABA_B$ agonist	Muscle spasticity	Several RCTs studies showed mixed results in reducing drinking	Drowsiness. At high doses more adverse events including fatigue, sleepiness, drowsiness, dizziness, dry mouth
Higher doses are being explored				

Ondansetron (8 μg/kg/day)	5-HT_3 antagonist	Nausea and vomiting	Two large single-site RCTs demonstrated efficacy of ondansetron (low dose of 8 μg/kg/day) in reducing drinking, particularly in a subgroup of alcohol-dependent patients. The first RCT showed that patients with early onset of alcoholism (25 years or younger) responded to ondansetron treatment. The second RCT did not replicate this finding, but did find that genetic polymorphisms of 5-HTT, 5-HT_{3A}, and 5-HT_{3B} genes showed a greater response to ondansetron	None reported in the two RCTs. FDA safety precaution warns that cardiac QT prolongation is possible at high doses
Prazosin/ doxazosin (16 mg/day)	Selective α-1 adrenergic antagonist	Hypertension and benign prostatic hyperplasia	Single-site RCTs have shown mixed effects. May work in a subgroup with a family history and high blood pressure	Drowsiness, dizziness, fatigue
ABT-436 (800 mg/day)	Vasopressin V1b antagonist	Not approved	Multisite RCT showed increase in % days abstinent. May work better in individuals reporting higher baseline levels of stress	Diarrhea
Aripiprazole (15–30 mg/day)	Partial agonist at D_2 and 5-HT_{1A} receptors, 5-HT_2 antagonist	Schizophrenia, bipolar disorder adjunct for major depression	Small human laboratory and clinical studies suggested efficacy for reducing drinking. However, a multisite RCT showed no efficacy at a 30 mg/day dose	Fatigue, insomnia, restlessness, somnolence, anxiety, and disturbance in attention. FDA Box Warning for suicidal thoughts and behaviors
LY 2940094 (40 mg/day)	Nociception (NOP) antagonist	Not approved	Multisite RCT showed reduced number of heavy-drinking days and increase in % days abstinent	Insomnia, anxiety, vomiting
LY 2196044 (250 mg/day)	Opioid antagonist	Not approved	Multisite RCT showed reduced number of drinks per day. May work better in individuals with repeat L DRD4 gene polymorphism	Gastrointestinal-related side effects similar to naltrexone and nalmefene
Medications that have shown poor efficacy in multisite alcohol pharmacotherapy clinical trials despite promising preliminary studies				
Quetiapine (300–800 mg/ day)	Blocks dopamine D_1 and D_2, 5-HT_{1A}, 5-HT_{2A}, histamine H_1, and adrenergic α_1 and α_2	Schizophrenia, manic episodes associated with bipolar 1 disorder, depressive episodes associated with bipolar disorder, adjunct for major depressive disorder	Despite promising results in preliminary human studies, quetiapine was not effective in several multisite and single site RCTs. In the multisite RCT, no promising subgroups of patients could be identified	Dizziness, dry mouth, dyspepsia, increased appetite, sedation, and somnolence. FDA Box Warning for suicidal thoughts and behaviors
Levetiracetam (1 to 2 g/day)	Activates GABA and glycine systems, inhibits glutamate AMPA, depresses sodium-calcium channel current, modulates synaptic vesicle protein 2A	Adjunct for myoclonic seizure, partial seizure, tonic-clonic seizure	Several single site and multisite RCTs trials showed no effect on reducing drinking	Fatigue. Fewer side effects than the anticonvulsants topiramate and zonisamide

(continued)

Table 1 (continued)

Medication (dose)	Site of action	FDA approval	Efficacy	Common side effects
Promising medications: ongoing studies				
Mifepristone (600 mg/day for 1 week)	Glucocorticoid antagonist	Pregnancy termination, adjunct for hyperglycemia and endometriosis	Reduced alcohol craving during laboratory session and reduced alcohol consumption during 1 week before and after human laboratory session	Well-tolerated with no serious adverse events
Oxytocin (nasal 40–48 IU/day)	Oxytocin receptor agonist	Labor induction, termination of pregnancy, adjunct in management of incomplete, or inevitable abortion	Reduces acute alcohol withdrawal symptoms. May work better in individuals with high levels of anxiety	Well-tolerated
Ibudilast (100 mg/day)	Nonselective phosphodiesterase inhibitor	Not approved	In subgroup analysis, individuals with elevated depression experienced reduced stimulant and mood-altering effects of alcohol	Well-tolerated
D-cycloserine (acute dose of 50 mg)	Partial agonist at glycine modulatory site of glutamate NMDA receptor	Tuberculosis, urinary tract infection	Reduced alcohol craving and decreased brain activation in the ventral and dorsal striatum	Well-tolerated

needed (one 18-mg tablet per day) if the patient perceived a risk of relapse. Patients taking nalmefene significantly reduced the number of days they drank heavily and the total amount of alcohol they consumed compared with the placebo group (8.2 versus 10.7 heavy-drinking days; 33.3 versus 45.5 g of alcohol per day, respectively). The design of the second 24-week RCT was similar to the first, with 718 alcohol-dependent patients recruited across 57 sites in Belgium, the Czech Republic, France, Italy, Poland, Portugal, and Spain (Gual et al. 2013). Again, patients taking nalmefene as needed showed a significant reduction in the number of heavy-drinking days and a reduction (nonsignificant) in the amount of alcohol they consumed compared with the placebo group (6.6 versus 7.5 heavy-drinking days; 30 versus 33 g of alcohol per day, respectively). Interestingly, a secondary analysis of a subgroup of the heaviest drinkers in the two trials showed even larger nalmefene treatment effects (van den Brink et al. 2013). A year-long RCT trial was also conducted in 675 alcohol-dependent patients across 60 sites in the Czech Republic, Estonia, Hungary, Latvia, Lithuania, Poland, Russia, Slovakia, Ukraine, and the United Kingdom (van den Brink et al. 2014). Although there was no significant difference in drinking outcomes between those taking nalmefene as needed and the placebo groups at 6 months, there was a significant reduction in heavy-drinking days and alcohol consumption in the nalmefene group at 12 months. Nalmefene was well-tolerated across the three studies, with the most common side effects being nausea, vomiting, fatigue, insomnia, and dizziness. Although the oral formulation of nalmefene is not yet available or approved for use in AUD in the United States, it is approved for use in alcohol dependence in Europe.

1.1.2 Varenicline

Molecular and Functional Mechanisms of Action

Varenicline is a partial agonist at the α4β2 and a full agonist at α7 nicotinic acetylcholine receptor (Mihalak et al. 2006). It has been approved by the FDA for smoking cessation. Recent evidence indicates that varenicline also attenuates drinking in alcohol-dependent individuals. In several human laboratory studies, varenicline reduced alcohol craving and the subjective reinforcing effects of alcohol in non-alcohol-dependent heavy-drinking smokers, heavy-drinking smokers and nonsmokers, and non-treatment-seeking alcohol-dependent individuals (McKee et al. 2009; Roberts et al. 2017a, b; Schacht et al. 2014). Interestingly, in one human laboratory study, varenicline was most effective in heavy drinkers with high levels of depressive symptoms, in both reducing alcohol craving and alcohol self-administration (Roberts et al. 2017b). Finally, Vatsalya et al. (2015) showed that varenicline decreases activity in the striato-cortico-limbic regions of the brain, which are associated with alcohol craving/urges in heavy drinkers.

Results of Clinical Trials

Several small RCTs of varenicline showed efficacy in problematic drinkers. Fucito et al. (2011) conducted an 8-week RCT of varenicline (titrated up to 2 mg per day) in 30 heavy-drinking smokers. Varenicline was effective in reducing alcohol craving,

resulting in fewer heavy-drinking days compared with those who received placebo. In a 16-week RCT with 64 heavy-drinking smokers, varenicline (2 mg per day) significantly reduced alcohol consumption compared with placebo (Mitchell et al. 2012b). In a 13-week RCT of 40 alcohol-dependent individuals, varenicline (2 mg per day) reduced alcohol craving compared with placebo, but drinking outcomes were similar (Plebani et al. 2013). However, smokers were less likely to report heavy drinking when taking varenicline compared with those taking placebo.

Results of these small RCTs were confirmed by a larger 13-week multisite RCT of varenicline (2 mg per day) in 200 alcohol-dependent individuals, approximately 40% of whom were smokers (Litten et al. 2013). Compared with the placebo group, the varenicline group had significantly fewer heavy-drinking days (37.9% each week versus 48.4%), drinks per day (4.4 versus 5.3), and drinks per drinking day (5.8 versus 6.8). Furthermore, varenicline also reduced alcohol craving. The efficacy was the same among smokers and nonsmokers. Notably, although the subgroup of alcoholic smokers was not seeking treatment for smoking, varenicline still reduced the number of cigarettes per day among smokers. A moderator analysis indicated that varenicline was most effective in individuals with less severe AUD and in those who reduced their smoking (Falk et al. 2015). Varenicline was well-tolerated. The most common adverse effects were nausea, abnormal dreams, and constipation. In another multisite RCT conducted in Sweden, 160 alcohol-dependent individuals were given either varenicline (2 mg per day) or placebo for 12 weeks (de Bejczy et al. 2015). In this study, there were no differences in self-reported drinking outcomes or reduction in smoking between the varenicline and placebo groups. However, there were significant reductions in alcohol craving, the number of reported AUD symptoms (measured using the Alcohol Use Disorders Identification Test [AUDIT]), and blood levels of phosphatidyl ethanol (PEth), a specific biomarker of alcohol consumption – all supporting an effect of varenicline on alcohol consumption.

The FDA recently removed the Box Warning about possible neuropsychiatric side effects on mood, behavior, or thinking when taking varenicline (https://www.fda.gov/Drugs/DrugSafety/ucm532221.htm). However, the FDA has issued a warning that varenicline may change the way patients respond to alcohol, affecting their ability tolerate its effects. Moreover, in rare accounts, seizures have been reported in patients taking varenicline (https://www.fda.gov/Drugs/DrugSafety/ucm436494.htm). However, none of these side effects were observed in the above RCTs.

1.1.3 Topiramate

Molecular and Functional Mechanisms of Action

Topiramate is an anticonvulsant approved by the FDA for treatment of seizures, migraines, and obesity (combined with phentermine for the latter indication). It has multiple pharmacologic effects in the brain. Topiramate antagonizes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, facilitates GABA activity, blocks L-type calcium channels, reduces voltage-dependent sodium channel activity, and inhibits carbonic anhydrase (Arnone 2005). In two human

laboratory studies, topiramate reduced the frequency of heavy drinking and the stimulating effects of alcohol in one study (Miranda et al. 2008) and alcohol craving in the other (Miranda et al. 2016).

Results of Clinical Trials

Several clinical trials showed topiramate was effective in reducing drinking in alcohol-dependent individuals, which was summarized by a recent meta-analysis (Blodgett et al. 2014). Johnson et al. (2003) first demonstrated the efficacy of topiramate in a single-site 12-week RCT with 150 alcohol-dependent individuals. Individuals receiving topiramate (up to 300 mg per day) had fewer drinks per day, more days abstinent, less heavy-drinking days, lower GGT, and less alcohol craving than those treated with placebo. Notably, unlike previous alcohol pharmacotherapy RCTs, this study did not require participants to be abstinent from alcohol before starting the trial. Therefore, the study assessed when abstinence began rather than how well it was maintained (i.e., abstinence initiation versus abstinence persistence). Side effects included dizziness, paresthesia, psychomotor slowing, memory or concentration impairment, nervousness, taste perversion, pruritus, and weight loss. The results of this study were replicated in a larger 14-week multisite RCT with 371 alcohol-dependent individuals (Johnson et al. 2007). Again, topiramate (titrated up to 300 mg per day) significantly reduced the number of heavy-drinking days, drinks per drinking day, and serum GGT levels and increased the number of days abstinent. The side-effect profile was similar to the original study.

Several smaller RCTs of topiramate have since followed. Knapp et al. (2015) conducted a single-site 14-week RCT in 21 alcohol-dependent individuals and found that topiramate (300 mg per day) significantly reduced the number of drinks per day, drinking days, and heavy-drinking days compared with placebo. Martinotti et al. (2014) conducted a low-dose topiramate (100 mg per day) RCT in 52 alcohol-dependent individuals and found it was effective in reducing daily alcohol consumption and alcohol craving and increasing the number of days abstinent. The side-effect profile for this lower dosage was more favorable than studies using 200 mg per day and 300 mg per day. In another study, Kranzler et al. (2014) conducted a 12-week RCT of topiramate (200 mg per day) in 138 alcohol-dependent individuals. Those receiving topiramate experienced fewer heavy-drinking days, reduced serum levels of GGT, and more abstinent days relative to placebo. Moreover, a post hoc analysis revealed that topiramate's increased efficacy was moderated by a single nucleotide polymorphism (SNP). Patients with the CC genotype of the rs2832407 GRIK1 gene encoding the glutamate kainate GluK1 receptor showed improvement in drinking outcomes, whereas the other genotypes (AC and AA genotype) did not show efficacy with topiramate. Notably, a previous small laboratory study with heavy drinkers showed that individuals with the CC genotype of the rs2832407 GRIK1 gene had significantly fewer topiramate-related side effects and lower topiramate blood concentrations (Ray et al. 2009). A two-site RCT currently is being conducted in alcohol-dependent European-Americans and African-Americans to see if these promising findings can be replicated, both in terms of efficacy and safety/tolerability (ClinicalTrials.gov NCT02371889).

Interestingly, Anthenelli et al. (2017) recently conducted a 12-week RCT of topiramate (200 mg per day) in 129 alcohol-abstinent (mean of ~6 months) alcohol-dependent male smokers (80% had other substance use disorders). In this population, topiramate was not effective in reducing relapse to alcohol or smoking or other drug use. Thus, topiramate may be more useful in preventing or reducing alcohol consumption in current drinkers than in preventing relapse to drinking in individuals with AUD who are abstinent. This finding was consistent with other positive RCTs (Johnson et al. 2003, 2007) where abstinence initiation rather than abstinence persistence was assessed.

As a final note, it appears that topiramate has gained acceptance with some large clinical care provider organizations, such as the Veterans Health Administration (Del Re et al. 2013). Nonetheless, research continues to explore other medications with similar mechanisms of action but with a more favorable safety profile, particularly regarding cognitive dysfunction (see zonisamide and levetiracetam below).

1.1.4 Zonisamide

Molecular and Functional Mechanisms of Action

Zonisamide is approved by the FDA as an adjunct treatment for partial seizures. Like topiramate, it has multiple molecular actions in the brain, including blocking voltage-sensitive sodium channels and T-type calcium channels, facilitating GABA activity, and serving as a weak inhibitor of carbonic anhydrase (Kothare and Kaleyias 2008). However, the side-effect profile of zonisamide appears to be more favorable than topiramate, with the most common being somnolence, anorexia, dizziness, decreased cognitive dysfunction, headache, nausea, and agitation/irritability (Kothare and Kaleyias 2008; https://www.accessdata.fda.gov/drugsatfda_docs/label/2006/020789s019lbl.pdf). In a human laboratory study of risky drinkers, zonisamide reduced alcohol craving and alcohol intake in a self-administration paradigm compared with placebo (Sarid-Segal et al. 2009).

Results of Clinical Trials

Several open-label trials (no placebo group) suggest that zonisamide may be effective in reducing alcohol consumption and craving in alcohol-dependent individuals (Knapp et al. 2010; Rubio et al. 2010). Zonisamide was well-tolerated in these studies. This preliminary work was followed by a 12-week RCT of zonisamide (up to 500 mg per day) in 40 alcohol-dependent individuals (Arias et al. 2010). Zonisamide significantly reduced the number of heavy-drinking days, drinks per week, and alcohol craving compared with placebo. It was well-tolerated with no serious side effects. In another RCT, 85 alcohol-dependent individuals were administered either zonisamide (400 mg per day), topiramate (300 mg per day), levetiracetam (200 mg per day), or placebo for 14 weeks (Knapp et al. 2015). Zonisamide and topiramate significantly reduced the percent of drinking days per week, drinks per day, and heavy drinking per week compared with placebo (results were slightly more favorable for topiramate). Levetiracetam was only effective in decreasing the percent of heavy-drinking days per week. Neurotoxicity and cognitive function were also evaluated for the three compounds. Individuals treated with

zonisamide or topiramate were more likely to experience impairments in verbal fluency and verbal working memory. Those treated with topiramate also had visual memory impairments, and were slower to recall events, compared with zonisamide. This indicates less severe side effects with zonisamide compared with topiramate. Levetiracetam did not produce any performance decreases on the neuropsychological tests.

Several ongoing trials are being conducted with zonisamide in veteran and non-veteran AUD patients with and without posttraumatic stress disorder (ClinicalTrials.gov NCT02368431, NCT01847469, NCT02901041, and NCT02900352).

1.1.5 Gabapentin

Molecular and Functional Mechanisms of Action

Gabapentin is approved by the FDA for the treatment of epileptic seizures, neuropathic pain, and restless legs syndrome. Its mechanism of action is thought to be related to its inhibition of voltage-gated calcium channels, which indirectly modulate GABA activity (Sills 2006). In a human laboratory study, Mason et al. (2009) demonstrated that gabapentin (1,200 mg per day) was effective in reducing alcohol craving and improving sleep quality.

Results of Clinical Trials

Several single-site RCTs of gabapentin have been conducted in alcohol-dependent patients. Furieri and Nakamura-Palacios (2007) conducted a 4-week RCT of gabapentin (600 mg per day) in 60 male alcohol-dependent patients from a Brazilian public outpatient drug treatment center. Gabapentin significantly reduced the number of drinks per day and number of heavy-drinking days and increased the percent of days abstinent. Brower et al. (2008) conducted a small 6-week RCT trial of gabapentin (titrated to 1,500 mg per day) in 21 alcohol-dependent individuals who also were diagnosed with insomnia. Gabapentin significantly delayed the onset of heavy drinking compared with placebo, but did not differ from the placebo group in improving insomnia. In another RCT, gabapentin (up to 1,200 mg per day for 39 days) was combined with flumazenil (20 mg per day for first 2 days) in 60 - alcohol-dependent patients (44 with relatively fewer and 16 with relatively more pretreatment alcohol withdrawal symptoms) (Anton et al. 2009). Individuals with more alcohol withdrawal symptoms before treatment had an increase in the percent of days abstinent and a longer delay to heavy drinking when taking combined gabapentin and flumazenil compared with placebo. On the other hand, those with fewer alcohol withdrawal symptoms before treatment did not differ in drinking outcomes compared with the placebo groups. In another RCT, 150 alcohol-dependent individuals received either 16 weeks of naltrexone (50 mg per day) or naltrexone (50 mg per day) combined with gabapentin (up to 1,200 mg per day for the first 6 weeks) or double placebo (Anton et al. 2011). During the first 6 weeks, the combined medication group experienced a longer delay to heavy drinking, less heavy-drinking days, and fewer drinks per drinking day than the group taking naltrexone alone or those receiving the placebo. In addition, the naltrexone/

gabapentin group reported significantly better sleep than the other two groups. Finally, Mason et al. (2014) conducted a 12-week RCT of gabapentin (900 mg per day and 1,800 mg per day) in 150 patients with alcohol dependence. Gabapentin significantly improved the rates of abstinence and no heavy drinking. The abstinence rate was 4.1% for the placebo group, 11.1% for the 900 mg group, and 17.0% for the 1,800 mg group, while the rate of no heavy drinking was 22.5% for the placebo group, 29.6% for the 900 mg group, and 44.7% in the 1,800 mg group. Gabapentin also showed a dose effect on alcohol craving, mood, and sleep. Gabapentin was well-tolerated with the most common side effects being fatigue, insomnia, and headaches.

Although gabapentin is considered to have no abuse potential, a recent report indicates that gabapentin potentially may be misused in substance abuse populations, especially those who abuse opioids (Smith et al. 2016). NIAAA currently is conducting a multisite RCT on enacarbil gabapentin, an extended-release prodrug (a medication that is metabolized into a pharmacologically active drug after administration) designed to increase its bioavailability (ClinicalTrials.gov: NCT02252536).

1.1.6 Baclofen

Molecular and Functional Mechanisms of Action

Baclofen is a $GABA_B$ agonist approved by the FDA for the treatment of muscle spasticity. Several human laboratory studies suggest that baclofen may affect alcohol drinking behavior by changing the subjective effects of alcohol (Evans and Bisaga 2009; Farokhnia et al. 2017; Leggio et al. 2013).

Results of Clinical Trials

Several RCTs have provided mixed results on the efficacy, optimal dose, and safety of baclofen. Addolorato et al. (2002) conducted a 4-week RCT of baclofen (30 mg per day) in 39 alcohol-dependent individuals. The results showed that baclofen increased abstinence rates and the number of days abstinent compared with placebo. The medication also reduced alcohol craving and state anxiety levels. The same group conducted a larger 12-week RCT of baclofen (30 mg per day) in 84 alcohol-dependent individuals with co-occurring liver cirrhosis (Addolorato et al. 2007). Individuals treated with baclofen experienced a higher rate of abstinence compared with placebo (71% versus 29%, respectively). Baclofen also significantly increased number of days abstinent and reduced alcohol craving. Baclofen was well-tolerated with no additional hepatic side effects. Indeed, there was an improvement of blood liver tests in the baclofen group versus placebo, most likely because of baclofen's effects in facilitating alcohol abstinence. In contrast, Garbutt et al. (2010) found no effect of baclofen (30 mg per day) in a 12-week RCT with 80 alcohol-dependent individuals. There were no differences in number of heavy-drinking days, percent of days abstinent, time to first drink, or time to relapse to heavy drinking between the baclofen and placebo groups. However, baclofen reduced the level of anxiety state, consistent with its mechanism of reducing the symptoms of alcohol withdrawal. Baclofen was well-tolerated with the most

common side effect being drowsiness. Morley et al. (2014) conducted a 12-week RCT with 42 alcohol-dependent individuals using 30 mg per day of baclofen, 60 mg per day of baclofen, or placebo. There were no differences in time to first heavy-drinking day, time to first drink, number of heavy-drinking days, drinks per drinking day, or percent of days abstinent between the baclofen and placebo groups. In a subgroup analysis, individuals who received 30 mg per day of baclofen and who had a lifetime or current anxiety disorder experienced a significant increase in the time to first drink and time to first heavy-drinking day. Again, the major side effect of baclofen was drowsiness. Finally, a small 12-week RCT was conducted to determine if baclofen (80 mg per day) was effective in treating alcohol-dependent individuals who also were smokers ($n = 30$) (Leggio et al. 2015). Baclofen, compared with placebo, significantly increased the number of days abstinent from alcohol and tobacco co-use, but did not significantly increase the number of days abstinent from alcohol use alone.

Two anecdotal reports showing that high doses of baclofen [up to 140 mg per day (Bucknam 2007) and up to 300 mg per day (Ameisen 2005)] resulted in abstinence from alcohol led researchers to wonder if it was more effective in these higher amounts. Muller et al. (2015) conducted a RCT with a high dose of baclofen (up to 250 mg per day) in 56 alcohol-dependent individuals. Those treated for 12 weeks with 250 mg per day had a greater abstinence rate (68.2% versus 23.8% for placebo) and more cumulative abstinence duration (67.8 versus 51.8 for placebo). No serious adverse events were observed. In contrast to these findings, Beraha et al. (2016) conducted a RCT of baclofen in 151 alcohol-dependent individuals who were administered a high dose (150 mg per day; 6 weeks titration, 10 weeks of high dose), a low dose (30 mg per day), or placebo. There were no differences in time to first relapse or abstinence rates among the three groups (although only 15.5% of those in the high dose group reached the 150 mg per day). The high dose group experienced more adverse events, particularly fatigue, sleepiness, drowsiness, dizziness, and dry mouth. In another multisite RCT, baclofen (180 mg per day) or placebo was administered to 320 alcohol-dependent individuals for 24 weeks (Reynaud et al. 2017). Although baclofen significantly reduced alcohol craving, there were no significant differences in percent of abstinence and drinks per day between the baclofen and placebo groups. However, in a subgroup analysis, baclofen was more effective than placebo in reducing drinking in individuals who were drinking heavily at the start of the study. There were no major adverse effects; the most common were somnolence, sleep disorders, asthenia, and dizziness.

The mixed results of these RCTs suggest that baclofen works in a subgroup of patients. In particular, it is conceivable that baclofen might be an effective medication in those patients with higher severity of alcohol dependence (Leggio et al. 2010). Whether higher doses are more effective remains unclear and controversial, although higher doses may have a greater risk of adverse events. For example, Rolland et al. (2015), in a study of 253 alcohol-dependent individuals, found a positive relationship between the dose of baclofen and the likelihood of major sedation. In addition, Boels et al. (2017) reported that baclofen, prescribed in high

doses, may lead to severe poisoning (cardiovascular, neurological metabolic, respiratory symptoms), particularly in patients with psychiatric illnesses. Currently, several RCTs are being conducted using baclofen, which may shed light on its efficacy, optimal dose (high versus low), safety, and possible subgroups who may respond more favorably to this medication (ClinicalTrials.gov NCT02835365, NCT 03034408, NCT01980706, NCT02723383, and NCT02596763).

1.1.7 Ondansetron

Molecular and Functional Mechanisms of Action

Ondansetron is a selective serotonin 5-HT_3 antagonist approved by the FDA for the treatment of nausea and vomiting. Several laboratory studies have shown that ondansetron reduces the desire to drink and augments the stimulating and sedating effects of alcohol (Johnson et al. 1993; Kenna et al. 2009; Swift et al. 1996).

Results of Clinical Trials

In a groundbreaking RCT, Sellers et al. (1994) administered 0.25 mg or 2.0 mg per day of ondansetron or placebo to 71 alcohol-dependent individuals. After 6 weeks of treatment, the 0.25 mg ondansetron group significantly decreased drinks per drinking day compared with placebo (35% versus 21% reduction) in people consuming ten or less drinks per drinking day at baseline. Interestingly, the daily 0.25 mg dose of ondansetron was more effective than the 2.0 mg dose. In a subsequent 11-week RCT, 271 alcohol-dependent individuals were randomized to receive 2 μg/kg, 8 μg/kg, or 32 μg/kg per day of ondansetron or placebo (Johnson et al. 2000). An equal number of patients with early (25 years of age or younger) and late (>25 years) onset of alcoholism were recruited. Ondansetron significantly reduced the number of drinks per day and the number of drinks per drinking day compared with placebo, but only in people with early onset of alcoholism. The 8 μg/kg dose of ondansetron was the most effective dose, increasing the percent of days abstinent and total abstinence compared with placebo. Ondansetron was well-tolerated with no serious adverse events. Kranzler et al. (2003) later confirmed these results by conducting an open-label trial of ondansetron (8 μg per kg per day) in 40 early and late-onset alcohol-dependent individuals. The early-onset individuals experienced a greater decrease in drinks per day, drinks per drinking day, and alcohol-related consequences compared with late-onset alcoholic individuals.

These clinical studies led researchers to question if a biological marker might be able to predict a response to ondansetron. In particular, Johnson et al. (2011) hypothesized that there might be a pharmacogenetic interaction between ondansetron and the *SLC6A4* gene. That gene encodes the serotonin transporter, 5-HTT, which is associated with several psychiatric disorders. The *SLC6A4* consists of two types of alleles, a long form (L) and a short form (S) consisting of 44 less base pairs. Johnson et al. (2011) conducted an 11-week RCT of ondansetron (8 μg/kg per day) in 283 dependent individuals randomized to three different genotypes: LL, LS, and SS genotypes of 5-HTT. Individuals with the LL genotype who received ondansetron had a significantly lower number of drinks per drinking day and a higher percent of days abstinent than those

treated with the LS/SS genotypes who received the medication or those who received placebo regardless of their genotype. The research team also discovered another functional single nucleotide polymorphism (T/G) rs1042173, in the 3′-untranslated region of the *SLC6A4* gene. Individuals with the LL/TT genotype experienced a significantly lower number of drinks per drinking day and higher percent of days abstinent than all other genotypes and treatment groups combined. In a later analysis from the same RCT, Johnson et al. (2013) found additional functional genetic polymorphisms in the *HTR3A* and *HTR3B* genes, including AC in the rs17614942 in the *HTR3B* gene and AG in the rs1150226 and GG in the rs1176713 portion of the *HTR3A* gene. Ondansetron was more effective in reducing the number of drinks per drinking day and the number of heavy-drinking days and increasing the percent of days abstinent in people carrying one or more of these genetic variants. Kenna et al. (2014a) conducted a laboratory study of ondansetron and sertraline in 77 non-treatment-seeking alcohol-dependent individuals. Consistent with Johnson et al. (2011), Kenna et al. (2014a) found a pharmacogenetic interaction between the LL genotype and ondansetron, but no effect was found for sertraline. Specifically, ondansetron was effective in reducing the amount of drinks per drinking day in people with the LL genotype. In a later analysis from the same human laboratory study, Kenna et al. (2014b) found that women (not men) who had the LL genotype and equal or greater than 7 exon III repeats on the dopamine receptor D4 gene (*DRD4*) experienced less alcohol intake after taking ondansetron (0.5 mg per day for 3 weeks).

Finally, ondansetron given in doses cited above was much lower than that required to treat nausea and vomiting (approximately 10 times less). The FDA has issued a safety warning for ondansetron, indicating it may increase the risk of abnormal electrical activity in the heart (https://www.fda.gov/Drugs/DrugSafety/ucm271913.htm). However, this side effect may not be evident at the low doses required to treat AUD.

NIAAA is currently supporting a two-site RCT of ondansetron to confirm these promising genetic findings of Johnson et al. (2011, 2013) (ClinicalTrials.Gov NCT02354703).

1.1.8 Prazosin and Doxazosin

Molecular and Functional Mechanisms of Action

Prazosin and doxazosin are both selective α-1 adrenergic antagonists approved by the FDA to treat hypertension and benign prostatic hyperplasia. Fox et al. (2011) conducted a human laboratory study on prazosin (16 mg daily) in 17 recently abstinent, treatment-seeking alcohol-dependent individuals. Following cue and stress exposure, the prazosin group significantly reduced alcohol craving and reported lower levels of anxiety and negative emotions compared with the placebo group.

Results of Clinical Trials

A 6-week study by Simpson et al. (2009) found that prazosin (16 mg per day), compared with placebo, reduced drinking days per week and drinks per week in

24 alcohol-dependent patients without posttraumatic stress disorder (PTSD). Subsequently, Simpson et al. (2015) conducted a small 6-week RCT of prazosin (16 mg per day) in 30 individuals with co-occurring alcohol dependence and PTSD. Individuals treated with prazosin experienced a reduction in the percent of drinking days and percent of heavy-drinking days compared with placebo. No significant differences were found between the prazosin and placebo groups in terms of the PTSD symptoms. This is surprising because prazosin has been shown to be effective in treating PTSD (Green 2014). The most common side effects of prazosin were drowsiness, dizziness on standing, and fatigue. Petrakis et al. (2016) later conducted a 13-week RCT of prazosin (16 mg per day) in 96 veterans with co-occurring alcohol dependence and PTSD. There were no significant differences between the prazosin and placebo groups in the drinking outcomes, alcohol craving, or the PTSD symptoms. The mixed results of these studies suggest that the medication may be effective only in subgroups. Raskind et al. (2016), for example, reported that prazosin worked best in reducing PTSD symptoms in patients who had a higher baseline standing blood pressure. Current studies are investigating this compound in people with comorbid alcohol dependence and PTSD (ClinicalTrials.gov NCT00585780 and NCT02226367).

Alcohol researchers also are investigating doxazosin, another α-1 adrenergic antagonist. The advantage of doxazosin over prazosin is that given its significantly longer half-life, doxazosin requires only once-a-day dosing compared with prazosin's two to three dosages per day; frequency of side effects is lower; and, unlike prazosin, doxazosin may be taken with or without food (Leggio and Kenna 2013). All these properties are important in RCTs and in clinical practice as they could potentially increase medication adherence. Kenna et al. (2015) conducted a 10-week RCT of doxazosin (up to 16 mg per day) in 41 alcohol-dependent individuals. In the main analysis, no significant differences were found in the drinking outcomes between the doxazosin and placebo groups. However, in a priori moderator analyses, doxazosin-treated individuals with a greater family history of alcoholism experienced a significant decrease in the number of drinks per week and in the number of heavy-drinking days per week compared with placebo-treated, high-family-history individuals. In a later analysis from the same RCT, Haass-Koffler et al. (2017) found that doxazosin, compared with placebo, reduced the number of drinks per week and heavy-drinking days per week in a subgroup of patients who had higher baseline standing blood pressure.

These studies suggest that precision medicine may be important in using α-1 adrenergic antagonists to treat AUD.

1.1.9 ABT-436 (Vasopressin V1b Receptor Antagonist)

Molecular and Functional Mechanisms of Action

Vasopressin, a peptide released from the hypothalamus, acts on the type 1b receptor (V1b) at the pituitary, activating the hypothalamic-pituitary-adrenal (HPA) axis, which, in turn, regulates the body's stress response (Milivojevic and Sinha 2017). In addition, vasopressin acts on the extra-hypothalamic stress system, especially in the

extended amygdala. Blocking the V1b receptors in various animal models has resulted in decreased alcohol intake (Edwards et al. 2011; Zhou et al. 2011).

Results of a Clinical Trial

In a recent 12-week multisite RCT, ABT-436, a novel selective V1b receptor antagonist, was evaluated in 150 alcohol-dependent individuals (Ryan et al. 2017). ABT-436 (titrated to 800 mg per day) significantly increased the percent of days abstinent compared with placebo (51.2 versus 41.6, respectively). The percent of heavy-drinking days was lower in people receiving ABT-436 compared with placebo, although the difference was not statistically significant. Other measures of drinking, alcohol craving, and alcohol-related consequences did not differ between the ABT-436 and placebo groups. However, in smokers, the compound significantly reduced the number of cigarettes consumed compared with placebo. In moderator analyses, individuals reporting higher baseline levels of stress responded better to ABT-436 than to placebo on drinking outcomes. ABT-436 was well-tolerated, with diarrhea (mild-to-moderate severity) being the most common side effect. Unfortunately, AbbVie, Inc. has recently discontinued development of this compound.

1.1.10 Aripiprazole

Molecular and Functional Mechanisms of Action

Aripiprazole is an atypical, antipsychotic medication approved by the FDA for the treatment of schizophrenia, bipolar disorder, and for use as adjunct treatment for major depression (Litten et al. 2016a). It has multiple pharmacological mechanisms in the brain, including acting as a partial agonist for the dopamine D_2 and serotonin $5\text{-}HT_{1A}$ receptors and as an antagonist to the $5\text{-}HT_2$ receptors (Fleischhacker 2005). Several human laboratory studies suggest that aripiprazole may affect drinking behavior. Kranzler et al. (2008) reported that aripiprazole (2.5 mg and 10 mg per day) increased the sedating effects of alcohol and, to a lesser degree, reduced the euphoric effects. In another human laboratory study, Voronin et al. (2008) reported that aripiprazole (up to 15 mg per day) reduced drinking in non-treatment-seeking alcohol-dependent individuals, but had no effect on self-reported "high," intoxication, or alcohol craving when compared with the placebo group. Finally, Myrick et al. (2010) showed that aripiprazole (15 mg per day) blunted alcohol-cue-induced brain activity in the right ventral striatum. Anton et al. (2017) recently completed a human laboratory study of aripiprazole (15 mg per day) in 99 non-treatment-seeking alcohol-dependent individuals. In a bar-lab setting, aripiprazole significantly decreased alcohol self-administration among individuals with low self-control and delayed the return to drinking in those with high impulsivity compared with placebo.

Results of Clinical Trials

Martinotti et al. (2009) conducted a 16-week RCT of aripiprazole (up to 15 mg per day) and naltrexone (50 mg per day) in 75 alcohol-dependent subjects. During treatment, the aripriprazole and naltrexone groups displayed similar reductions on

measures of abstinence, percent days abstinent and number of heavy drinking days. However, patients treated with aripiprazole remained abstinent for a longer period of time than those treated with naltrexone. Anton et al. (2008) conducted a 12-week multisite RCT of aripiprazole with mixed results. Aripiprazole (titrated up to 30 mg per day) was effective in reducing the number of drinks per drinking day compared with the placebo group and reduced CDT at weeks 4 and 8. However, the aripiprazole group did not differ from the placebo group in percent of days abstinent, number of heavy-drinking days, and time to first drinking day. The authors postulated that these lackluster results possibly were related to dose-related attrition, as the aripiprazole group had a higher dropout rate than the placebo group, especially with the 30 mg dose. The most common side effects from aripiprazole were fatigue, insomnia, restlessness, somnolence, anxiety, and disturbances in attention. It has been suggested that aripiprazole may be efficacious at lower doses (15 mg per day). NIAAA is currently supporting an aripiprazole RCT in individuals with AUD and bipolar disorder, comparing the 15 and 30 mg daily doses (NCT02918370).

1.1.11 LY 2940094 (Nociceptin Receptor Antagonist)

Molecular and Functional Mechanisms of Action

The nociceptin (NOP) receptor (formerly known as opioid receptor-like) belongs to the opioid receptor family. Several studies have shown that targeting this receptor modifies alcohol drinking behavior in animal models (Aziz et al. 2016; Ciccocioppo et al. 2004; Economidou et al. 2008, 2011). Recently, Lilly Research Laboratories synthesized a NOP antagonist, LY2940094, that demonstrated an antidepressant effect in individuals with major depressive disorder (Post et al. 2016a). In addition, Rorick-Kehn et al. (2016) reported that LY2940094 was effective in decreasing alcohol intake in animal models bred to show a preference for alcohol.

Results of a Clinical Trial

Post et al. (2016b) conducted an 8-week multisite RCT trial of LY2940094 (40 mg per day) in 88 alcohol-dependent individuals. LY2940094 reduced the number of heavy-drinking days compared with placebo (−24.5% versus −15.7%, respectively) and increased the percent of days abstinent (9.1% versus 1.9%, respectively). Although the primary endpoint, drinks per day, did not differ between the two groups in the full sample, the compound did reduce drinks per day in two subgroups: those who drank less at baseline and women. Because most of the women had lower baseline drinking, it was difficult to determine which moderator had the most effect. The compound was well-tolerated with no serious adverse effects. The most common side effects were insomnia, anxiety, and vomiting. Currently, BlackThorn Therapeutics has licensed the compound from Lilly for further development.

1.1.12 LY2196044 (Opioid Receptor Antagonist)

Molecular and Functional Mechanisms of Action

The novel compound LY2196044, synthesized by Eli Lilly and Company, is an opioid receptor antagonist at the mu, kappa, and delta receptors. This compound has been shown to reduce drinking in animal models (Wong et al. 2014) through a mechanism similar to naltrexone and nalmefene.

Results of a Clinical Trial

In a 16-week multisite trial in 375 alcohol-dependent individuals, LY2196044 (up to 250 mg per day) significantly reduced the number of drinks per day from baseline compared with placebo (−5.4 versus −4.7, respectively) (Wong et al. 2014). However, the number of heavy-drinking days and percent of days abstinent did not differ significantly between the two groups. In a subgroup analysis, LY2196044 significantly improved the drinking outcomes in people with the dopamine receptor type 4 (*DRD4*) gene carrying tandem repeat L (which occurred in 39% of the subjects), compared with L carriers who received only the placebo. LY2196044-treated individuals had more gastrointestinal-related adverse events than did placebo-treated individuals, a finding similar to other opioid antagonists, such as naltrexone and nalmefene.

1.2 Medications That Have Shown Poor Efficacy in Multisite Alcohol Pharmacotherapy Clinical Trials Despite Promising Preliminary Studies

1.2.1 Quetiapine

Molecular and Cellular Mechanisms of Action

Quetiapine is an atypical antipsychotic medication approved by the FDA for treatment of schizophrenia, manic episodes associated with bipolar I disorder, depressive episodes associated with bipolar disorder, and as an adjunct treatment for major depression (Litten et al. 2016a). Quetiapine has multiple actions in the brain, blocking the serotonin 5-HT_{1A} and 5-HT_{2A} receptors, the dopamine D_1 and D_2 receptors, the histamine H_1 receptors, and the adrenergic α_1 and α_2 receptors (Ray et al. 2010). Two human laboratory studies have found that non-treatment-seeking alcohol-dependent individuals treated with quetiapine (400 mg per day) experienced reduced alcohol craving and alcohol-induced sedation and impulsivity, compared with placebo (Moallem and Ray 2012; Ray et al. 2011).

Results of Clinical Trials

In three open-label studies, alcohol-dependent individuals who were treated with quetiapine improved their drinking outcomes (Martinotti et al. 2008; Monnelly et al. 2004; Sattar et al. 2004). Kampman et al. (2007) conducted a 12-week pilot RCT of quetiapine (400 mg per day) in 61 Type A and Type B alcohol-dependent individuals. [Type A alcoholics are characterized by late age of onset of problem

drinking, low severity of alcohol dependence, few childhood risk factors, less concomitant psychopathology, and reduced drug use. In contrast, Type B alcoholics are characterized by early age of onset of alcohol problems, high severity of alcohol dependence, polydrug use, and a high degree of concomitant psychopathology (Babor et al. 1992).] Quetiapine-treated Type B alcoholic individuals experienced fewer days of drinking and days of heavy drinking and less alcohol craving than Type B alcoholic individuals treated with placebo. In contrast, among the Type A alcoholic individuals, there were no differences between the quetiapine and placebo groups.

To confirm these findings, Litten et al. (2012) conducted a multisite 12-week RCT of quetiapine (titrated to 400 mg per day) in 224 alcohol-dependent individuals. Surprisingly, there were no differences between the quetiapine and placebo groups in percent of heavy-drinking days, drinks per day, drinks per drinking day, percent of days abstinent, and percent of abstinent individuals. In a subgroup analysis, Type B alcoholic individuals, regardless of whether they took quetiapine or placebo, did not differ in drinking outcome. As expected, quetiapine improved sleep and symptoms of depression. Quetiapine was well-tolerated, with the most common side effects being dizziness, dry mouth, dyspepsia, increased appetite, sedation, and somnolence. This study was followed by another 12-week RCT of quetiapine (titrated to 600 mg per day) in 90 alcohol-dependent individuals diagnosed with comorbid bipolar I or II disorders and depressed or mixed mood state (Brown et al. 2014). The quetiapine group did not differ from the placebo group in drinks per day, percent of days abstinent, drinks per drinking day, and percent of heavy-drinking days. Finally, in another 12-week RCT, Stedman et al. (2010) found that quetiapine (300–800 mg per day, flexible dosing) when added as an adjunct therapy with lithium or divalproex, was no better than placebo in reducing alcohol use in 362 individuals with comorbid alcohol dependence and bipolar I disorder. Together, these RCTs do not support the use of quetiapine for AUD.

1.2.2 Levetiracetam

Molecular and Functional Mechanisms of Action

Like topiramate and zonisamide, levetiracetam is approved by the FDA to treat seizures, yet it has fewer reported side effects (Litten et al. 2016a). It targets multiple mechanisms in the brain, including activating the GABA and glycine systems, inhibiting glutamate AMPA receptors, decreasing sodium/calcium channel currents, and modulating the synaptic vesicle protein 2A (Abou-Khalil 2008; De Smedt et al. 2007, Fertig et al. 2012).

Results of Clinical Trials

Two open-label trials indicated that levetiracetam reduced alcohol consumption in alcohol-dependent individuals (Mariani and Levin 2008; Sarid-Segal et al. 2008). This was followed by multiple RCTs. As mentioned previously (in the topiramate and zonisamide sections), Knapp et al. (2015) conducted a 14-week single-site RCT with zonisamide (400 mg per day), topiramate (300 mg per day), and levetiracetam

(2,000 mg per day) in 85 alcohol-dependent individuals. Levetiracetam showed less efficacy than zonisamide and topiramate in reducing drinking (only reducing the number of heavy-drinking days compared with placebo). Unlike zonisamide and topiramate, levetiracetam showed no cognitive impairment. This confirmed an earlier study by Gomer et al. (2007) that also found no cognitive impairment from levetiracetam. Fertig et al. (2012) conducted a 16-week multisite trial of levetiracetam (2,000 mg per day) in 130 alcohol-dependent individuals. There were no differences on any drinking outcomes (percent of heavy-drinking days, drinks per day, drinks per drinking day, or percent of days abstinent) between the levetiracetam and the placebo groups. The medication was well-tolerated, with fatigue being the only significant side effect. In another 16-week multisite RCT of levetiracetam (2,000 mg per day) in 201 recently detoxified alcohol-dependent individuals, Richter et al. (2012) found the compound did not affect the rate/time of relapse compared with the placebo. In addition, Mitchell et al. (2012a) conducted a 6-week single-site RCT of levetiracetam in 46 moderate-to-heavy drinkers. Individuals were given a 500–1,000 mg dose per day, a 1,000–2,000 mg dose per day, or placebo. Levetiracetam had no effect on drinking outcome. Interestingly, individuals who initially were drinking less actually experienced an increase in drinking with levetiracetam, compared with placebo. Thus, these RCTs indicate that levetiracetam has very limited efficacy, if any, for treating AUD individuals.

1.3 Promising Medications: Ongoing Human Studies

1.3.1 Mifepristone

Molecular and Functional Mechanisms of Action

Mifepristone is a glucocorticoid receptor antagonist approved by the FDA for terminating pregnancy (together with misoprostol) and for treating hyperglycemia in patients with Cushing syndrome and type 2 diabetes mellitus and endometriosis. Alterations in the brain glucocorticoid system are believed to drive compulsive-like alcohol consumption in rats (Richardson et al. 2008; Simms et al. 2012; Vendruscolo et al. 2012). Recently, Vendruscolo et al. (2015) tested the efficacy of mifepristone in both rats and humans. Mifepristone reduced alcohol intake in alcohol-dependent rats but not in non-dependent rats. Vendruscolo et al. (2015) then tested the medication (600 mg daily for 1 week) in a laboratory setting in 56 non-treatment-seeking alcohol-dependent people. Mifepristone significantly reduced alcohol-induced craving during the laboratory session and reduced alcohol consumption during the 1-week treatment phase and 1-week posttreatment phase compared with placebo. There were no serious adverse effects, and there were no differences in the type or severity of adverse events during treatment between the mifepristone and placebo groups. Currently, human laboratory and RCT studies are evaluating the efficacy and safety of mifepristone (ClinicalTrials.gov: NCT02243709 and NCT02179749).

1.3.2 Oxytocin

Molecular and Functional Mechanisms of Action

Oxytocin is a nine-amino acid polypeptide hormone approved by the FDA for inducing labor and terminating pregnancy and as an adjunctive therapy in the management of incomplete or inevitable abortion. It also plays a role in the brain's reward and stress systems, as well as in networks that have a role in social affiliations, learning, and memory. Recent studies show that oxytocin affects alcohol drinking behavior in animal models (Lee et al. 2016; Lee and Weerts 2016). In particular, King et al. (2017) demonstrated reduced alcohol consumption in different models of alcohol self-administration in mice, and Peters et al. (2016) reported that oxytocin inhibited alcohol consumption and alcohol-induced dopamine release in the nucleus accumbens of rats. A pilot RCT of oxytocin (48 IU per day, intranasal) was conducted in 11 alcohol-dependent individuals admitted for medical detoxification (Pedersen et al. 2013). Oxytocin was more effective than placebo in reducing craving and the symptoms of alcohol withdrawal. Mitchell et al. (2016) conducted a human laboratory study of oxytocin (40 IU, intranasal) in 32 non-treatment-seeking individuals with alcohol abuse. Subjects receiving oxytocin had significant improvements in social perception, compared with those receiving placebo, but there was no effect on alcohol-induced craving. However, in a subgroup analysis, oxytocin reduced alcohol craving in people with higher levels of attachment anxiety and increased alcohol craving in those with lower levels of attachment anxiety. Several human laboratory studies are currently underway to study the effects of oxytocin in AUD individuals (ClinicalTrials.gov NCT03046836, NCT02407340, and NCT02711189).

1.3.3 Ibudilast

Molecular and Functional Mechanisms of Action

Ibudilast (AV-411) is a nonselective phosphodiesterase inhibitor known to suppress glial cell activation and neuroinflammation (Ledeboer et al. 2007). Bell et al. (2013) showed that ibudilast reduced drinking in multiple animal models of alcohol dependence. In a crossover human laboratory study, ibudilast (100 mg per day) was no better than placebo in reducing subjective measures such as alcohol craving, stimulation, sedation, positive mood, and negative mood (Ray et al. 2017). However, ibudilast, compared with placebo, was associated with improvements in mood during stress- and alcohol-cue exposure. In a subgroup analysis, individuals with elevated depression had reduced stimulant and mood-altering effects of alcohol when given ibudilast, compared with those receiving placebo.

1.3.4 D-Cycloserine

Molecular and Functional Mechanisms of Action

D-cycloserine is a partial agonist at the glycine modulatory site of the glutamate NMDA receptor. It is FDA approved for the treatment of tuberculosis and urinary tract infection.

The compound is also thought to enhance learning, memory, and decision making because of its action on the NMDA receptor (Kelley 2004; Scholl et al. 2014). In addition, D-cycloserine has been shown to reduce alcohol intake in rats (Seif et al. 2015). Several human studies have been conducted with D-cycloserine to determine if the medication enhances cue-exposure therapy in alcohol-dependent and problem-drinking individuals. Watson et al. (2011) conducted a small human laboratory study in 16 abstinent alcohol-dependent individuals. D-cycloserine (single dose of 250 mg) did not differ from placebo in reducing the alcohol-induced cue response. However, because a high percentage of individuals had little or no response to the cue exposure, it was difficult for D-cycloserine to actually show an effect. In another human laboratory study, Hofmann et al. (2012) found that D-cycloserine (50 mg) increased alcohol craving compared with placebo in 20 non-treatment-seeking problem drinkers during an alcohol-cue paradigm. In contrast, MacKillop et al. (2015) later reported that D-cycloserine (50 mg) lowered cue-elicited craving for alcohol in 37 treatment-seeking AUD individuals. In addition, D-cycloserine reduced drinking during the 1-week interval between the cue-extinction paradigms. Finally, Kiefer et al. (2015) conducted a human laboratory study of D-cycloserine (50 mg) in 76 recently detoxified abstinent alcohol-dependent individuals. Using functional magnetic resonance imaging (fMRI), D-cycloserine, compared with placebo, decreased brain activation in the ventral and dorsal striatum, areas of the mesolimbic system associated with addiction. Further studies are needed to validate the efficacy of this compound for the treatment of AUD.

1.4 Other Promising Medications (Preclinical or Theoretical)

In addition to the medications above, NIAAA is supporting initial human studies of compounds that have shown promise in preclinical studies and/or have rational theory for efficacy in alleviating drinking in AUD individuals. These include the following medications along with their cllinicaltrials.gov number: PF-5190457 (ghrelin receptor inverse agonist) (NCT02707055), *N*-acetylcysteine (NAC) (precursor to glutathione) (NCT02966873), guanfacine (α_{2A} adrenoceptor agonist) (NCT02164422 and NCT03137082), minocycline (inhibitor of 5-lipoxygenase) (NCT02187211), citicoline (biosynthesis of phosphatidylcholine) (NCT02582905), dutasteride (5α-reductase inhibitor) (NCT01758523), pregabalin (inhibitor of voltage-gated calcium channel) (NCT02884908), and kudzu extract (mechanism unknown) (NCT03099590).

2 Final Remarks

From this review, it is clear that advances are being made in developing medications to treat AUD, and researchers are exploring numerous targets involved in alcohol-seeking and drinking behavior. Although progress has been made, the challenge over the next decade will be to develop medications that are more effective than the current ones and without serious side effects. To accomplish this, NIAAA has identified two long-range goals to increase the effectiveness of alcohol treatment

medications (Litten et al. 2016b): (1) advancing precision medicine and (2) discovering more effective targets.

Advancing precision medicine (or personalized medicine) is an essential step in identifying and targeting specific phenotypes that are most likely to respond favorably to a given medication. Although precision medicine is in its early stages, progress already is being made, especially in pharmacogenetics (Garbutt et al. 2014; Jones et al. 2015; Sun et al. 2016; see topiramate and ondansetron sections above). Still, given the complexities of AUD, it is doubtful that one factor, such as a person's genetic makeup, will be sufficient to predict a positive outcome for that individual (Litten et al. 2015). Most likely, multiple factors will need to be considered to successfully "match" an individual to a specific medication. Such factors include biomarker signatures from the various "-omics," including epigenomics, transcriptomics, proteomics, and metabolomics (Litten et al. 2016b). Other factors include biomarkers from brain imaging and electrophysiological variations, as well as patients' characteristics, such as demographics, drinking patterns, family history, AUD severity, and psychiatric/medical comorbidity. Using this "treatment fingerprint," an algorithm then could be established to describe a specific set of rules for matching individuals with medications. NIH's new groundbreaking initiative, the "All of Us Program" (formerly known as the Precision Medicine Initiative) (https://allofus.nih.gov), offers a tremendous resource for this. The program is drawing together data on more than 1 million individuals. It will be useful not only for identifying different phenotypes and genotypes but also will enable scientists to test new information and computational approaches for deciphering the heterogeneity of complex diseases like AUD.

A second long-range goal in discovering medications is to identify targets in the brain that will be effective across the multiple phenotypes of AUD. During the past two decades, scientists have focused their research on targets that affect craving and the urge/desire to drink. So far, these efforts have produced alcohol treatment medications that have only small effect sizes (Zindel and Kranzler 2014). As we begin to better understand the mechanisms underlying brain function, this undoubtedly will lead to new druggable targets. To help accomplish this goal, NIH has initiated several major programs, including the "Brain Research through Advancing Innovative Neurotechnologies (BRAIN) initiative." Supporting research on innovative technologies will enable us to examine how individual brain cells and neural circuits interact to produce specific behaviors, such as AUD, which can disrupt normal function (https://www.braininitiative.nih.gov/).

Another potential target involves the enzymes implicated in alcohol metabolism. We know that a genetic variant of the alcohol dehydrogenase gene (*ADH1B*2*) results in a more rapid alcohol metabolism, elevating acetaldehyde levels in the body (Hurley and Edenberg 2012). Because of the toxic and aversive actions of acetaldehyde, people with this genetic variant, which is common in Asian populations, are less likely to drink heavily and to become dependent on alcohol (Hurley and Edenberg 2012). Disulfiram, the first medication approved by the FDA for use in AUD, uses a similar mechanism of action, increasing acetaldehyde levels and making it uncomfortable to drink alcohol. However, disulfiram often is not

effective because patient adherence tends to be low, probably because this medication does not reduce alcohol craving (Johnson 2008). One strategy is to develop a compound that interferes with the alcohol metabolism, producing the desired aversive effect while, at the same time, reducing craving for alcohol (Diamond and Yao 2015). Another strategy is to produce a long-lasting compound that is active for weeks or perhaps months, eliminating the need to take daily dosages.

And finally, developing a rational, systematic way to identify druggable targets will be vital in the hunt for new medications. Currently, more than 30 targets have been identified that appear to influence alcohol-seeking and drinking behavior (Litten et al. 2016b). To better understand these targets, we need to know their role in causing and/or maintaining problematic drinking, whether they are related or independent of each other, and how they fit within the different domains of AUD (e.g., incentive salience/reward, negative affect/emotionality, and cognitive function) to produce the wide variety of responses to alcohol that we see across the population. It is particularly important to identify how these targets interrelate with molecular pathways and other brain circuits. One approach is to develop, integrate, and data mine biomolecular and cellular networks to discover druggable targets (Hopkins 2008; Masoudi-Nejad et al. 2013; Yildirim et al. 2007). Those networks are highly complex and include gene-gene, gene-protein, and protein-protein interactions, metabolic differences, and variations in gene expression and regulatory networks (Gebicke-Haerter 2016; Robinson and Nielsen 2016; Tang et al. 2013). Researchers examining other complex disorders are using this approach, including cancer, endocrine disorders, Huntington's disease, mood disorders, and schizophrenia (Collier et al. 2016; Morrow et al. 2010; Pirhaji et al. 2016; Yildirim et al. 2007). Many believe complex diseases like AUD cannot be effectively treated with one target–one medication; instead, successful treatment depends on multiple targets and combinations of medications. To date, there is a paucity of clinical studies where combined pharmacotherapies have been tested in AUD (Lee and Leggio 2014). To facilitate this line of research in the alcohol field, NIAAA recently issued a guide, *Development, Integration, and Data Mining of Biomolecular and Cellular Networks for Discovering Druggable Targets for Alcohol Use Disorder and Alcohol-Induced Organ Damage* (https://grants.nih.gov/grants/guide/notice-files/NOT-AA-17-007.html).

Ultimately, the success of our medications development program rests on our ability to get people into treatment. In any given year, less than 10% of individuals with AUD are offered or seek treatment (Grant et al. 2015). This meager rate would not be acceptable if these individuals were affected by cancer or another chronic medical condition. AUD is a chronic brain disorder. All patients suffering from AUD need to receive adequate treatment. By providing addiction-oriented education and training during medical school, clinical training, and beyond, we can help physicians better understand the pharmacotherapy options available to them, making AUD medications a routine part of standard practice.

In summary, the goals outlined above will be challenging but certainly are attainable. The NIAAA, the alcohol research community, the pharmaceutical industry, the clinicians, and the patients themselves all have a role in identifying, developing, and

implementing the next generation of medications. By developing more effective medications, with few side effects, and identifying the patients who will benefit the most from these treatments, we can provide clinicians with the tools they need to treat this devastating disorder, providing relief for patients and their families, and markedly improving public health and safety.

Acknowledgement The authors thank Barbara Vann of CSR, Incorporated for her review, edits, and thoughtful comments.

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Correction to: $GABA_A$ Receptor Subtype Mechanisms and the Abuse-Related Effects of Ethanol: Genetic and Pharmacological Evidence

Cassie M. Chandler, John S. Overton, Daniela Rüedi-Bettschen, and Donna M. Platt

Correction to:
Chapter "$GABA_A$ Receptor Subtype Mechanisms and the Abuse-Related Effects of Ethanol: Genetic and Pharmacological Evidence" in: C. M. Chandler et al., Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2017_80

The chemical name appearing in the first column of Table 1 on the 3rd row from bottom of the table is wrong. It should read as *tert*-Butyl 8-ethynyl-5,6-dihydro-5methyl-6-oxo-4H-imidazo[1,5-a[1,4]benzodiazepine-3-carboxylate. Further in section 4.2.1 on the third line of the second paragraph the word appears incorrect as "abercarnil". It should be "abecarnil". In the first reference in the list, the word appears incorrect as "Babrb1". It should be "Gabrb1". In the reference, Wafford KA, Whiting PJ, Kenp JA (1993b) Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant γ-aminobutyric acidA receptor subtypes. Mol Pharmacol 43:240–244, the name of the third author is incorrect. It should read as Kemp JA. The original chapter was corrected.

The updated online version of this chapter can be found at https://doi.org/10.1007/164_2017_80

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*, Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_190

Correction to: Presynaptic Ethanol Actions: Potential Roles in Ethanol Seeking

David M. Lovinger

Correction to:
Chapter "Presynaptic Ethanol Actions: Potential Roles in Ethanol Seeking" in: D. M. Lovinger,
Handbook of Experimental Pharmacology,
https://doi.org/10.1007/164_2017_76

The Acknowledgements unfortunately went missing to be included in the chapter. It has been included now. This work was supported by the Division of Intramural Clinical and Biological Research of the National Institute on Alcohol Abuse and Alcoholism, project ZIA AA000407. The original chapter has been corrected.

The updated online version of this chapter can be found at
https://doi.org/10.1007/164_2017_76

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_189

Correction to: Chronic Alcohol, Intrinsic Excitability, and Potassium Channels: Neuroadaptations and Drinking Behavior

Reginald Cannady, Jennifer A. Rinker, Sudarat Nimitvilai, John J. Woodward, and Patrick J. Mulholland

Correction to:
Chapter "Chronic Alcohol, Intrinsic Excitability, and Potassium Channels: Neuroadaptations and Drinking Behavior" in: R. Cannady et al., Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2017_90

In section 8.1 on the 10th line in first paragraph the reference citation Mateos-Aparicio et al. 2014 is incorrect. The correct citation is Rinker et al. 2017. The original chapter was corrected.

The updated online version of this chapter can be found at https://doi.org/10.1007/164_2017_90

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*, Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_192

Correction to: Innate Immune Signaling and Alcohol Use Disorders

Leon G. Coleman, Jr. and Fulton T. Crews

Correction to:
Chapter "Innate Immune Signaling and Alcohol Use Disorders" in: L. G. Coleman, Jr. and F. T. Crews, Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2018_92

In the second paragraph of section 1.3 on line 27, the text appears incorrect as glutamatergic and neurons. It should read as glutamatergic and GABAergic neurons. The original chapter was corrected.

The updated online version of this chapter can be found at https://doi.org/10.1007/164_2018_92

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*, Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_193

Correction to: Transcriptional Regulators as Targets for Alcohol Pharmacotherapies

Antonia M. Savarese and Amy W. Lasek

Correction to:

Handbook of Experimental Pharmacology,
https://doi.org/10.1007/164_2018_101

Figure 1 was published incorrectly in this chapter. The original chapter was corrected. The correct Figure 1 is

The updated online version of this chapter can be found at
https://doi.org/10.1007/164_2018_101

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_194

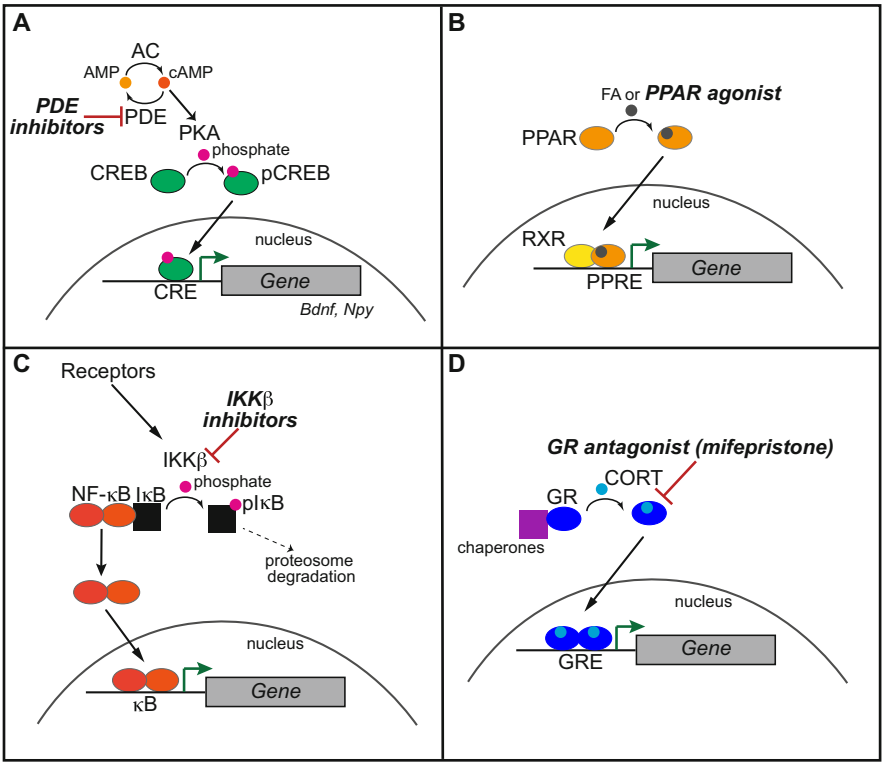

Fig. 1 Simplified diagram of transcriptional pathways and targets for intervention for alcohol use disorder (AUD) treatment. (**a**) The cAMP-responsive element binding protein (CREB) pathway. Adenylyl cyclase (AC) produces cAMP from AMP, activating protein kinase A (PKA). CREB is phosphorylated (pCREB) by several kinases, one of which is PKA. Once phosphorylated, CREB translocates to the nucleus and binds to cAMP-responsive elements (CRE) in the DNA to activate transcription of genes associated with AUD such as *Bdnf* and *Npy*. One method to activate CREB is to use compounds that inhibit the phosphodiesterases (PDEs) that hydrolyze cAMP, thus increasing cAMP levels and activating PKA. PDE inhibitors reduce alcohol consumption in animal models of AUD. (**b**) The peroxisome proliferator-activated receptor (PPAR) signaling pathway. PPARs are activated by their endogenous ligands, fatty acids (FA), or by synthetic agonists such as the thiazolidinediones and fibrates. Upon ligand binding, PPARs translocate to the nucleus and interact with retinoid X receptor (RXR) at peroxisome proliferator response elements (PPREs) to regulate gene transcription. PPAR agonists reduce alcohol consumption in animal models of AUD. (**c**) The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. NF-κB exists as a dimer of different subunits and is complexed with an inhibitory molecule, inhibitor κB (IκB) in the cytosol. Activation of various receptors leads to activation of IκB kinase (IKKβ) and phosphorylation of IκB. This event targets IκB for degradation, releasing NF-κB for translocation to the nucleus to regulate gene expression at κB elements. IKKβ inhibitors reduce alcohol consumption in mice. (**d**) Glucocorticoid receptor (GR) pathway. GR is held in the cytosol by chaperone proteins. Once bound to its ligand, cortisol (in humans/nonhuman primates) or corticosterone (in rodents) (CORT), GR translocates to the nucleus and binds to glucocorticoid response elements (GREs) to regulate gene transcription. The GR antagonist mifepristone has shown efficacy in reducing alcohol consumption in rodents and humans

Correction to: Advancing Pharmacotherapy Development from Preclinical Animal Studies

Mark Egli

Correction to:
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Handbook of Experimental Pharmacology,
https://doi.org/10.1007/164_2017_85

In the 7th line from the bottom of the Abstract the word nociception appears incorrect. It should read nocioceptin. In section 3.4 on the third line of 2nd paragraph the word nociception appears incorrect. It should read nocioceptin. The original chapter was corrected.

The updated online version of this chapter can be found at
https://doi.org/10.1007/164_2017_85

K. A. Grant, D. M. Lovinger (eds.), *The Neuropharmacology of Alcohol*,
Handbook of Experimental Pharmacology 248, https://doi.org/10.1007/164_2018_191