Neurotrophic Factors and Ethanol Neurotoxicity

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Contents

Abstract

Neurotrophins are essential for the growth, differentiation, and survival of neurons during development and in the adult. Considerable data have accumulated over the last few decades implicating classical neurotrophins, Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), and Neurotrophins 3 and 4 (NT-3/4), in many aspects of ethanol addiction, neurotoxicity, and repair after ethanol withdrawal. Genetic screens in *Drosophila* have identified novel neurotrophic factors and signaling intermediates involved in acute tolerance and pharmacodynamic adaptation to prolonged exposure in rodent. Ethanol modulates neurotrophic factor expression in vivo in a time- and

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region-specific fashion. Ethanol inhibits neurotrophin signaling acutely in vivo and in vitro in many brain regions. Conversely, acute and chronic ethanol exposure can upregulate neurotrophin-associated signaling pathways, particularly in brain nuclei associated with anxiety and addiction. Cell death induced by high concentrations of ethanol can be mitigated by exogenous neurotrophins indicating that neurotrophin induction in vivo may also be neuroprotective but ultimately fails over time. Neurotrophin levels in serum and plasma of patients with alcohol use disorders are dynamic and may serve as a surrogate for central nervous system levels. The kinetics suggest that increased levels during withdrawal may be involved in repair, but these analysis are complicated by genetic polymorphisms and the blood component analyzed, particularly with BDNF which is polymorphic in human populations and also produced by platelets. Neurotrophins are intricately involved in pharmacodynamic compensation with prolonged ethanol exposure, addiction-related plasticity, and neurotoxicity, but considerable work remains to be performed and replicated. Recent pharmacological advances targeting neurotrophins and neurotrophin signaling may ultimately be useful for treating ethanol-induced neurodegeneration and aberrant plasticity associated with addiction.

List of Abbreviations

AD	Alcohol dependence
ALK	Anaplastic lymphoma kinase
ARND	Alcohol-related neurodevelopmental disorders
BAC	Blood alcohol concentration
BDNF	Brain-derived neurotrophic factor
BFCN	Basal forebrain cholinergic nuclei
cAMP	3′-5′ cyclic adenosine monophosphate
ChAT	Choline acyltransferase
E	Embryonic
ERK	Extracellular signal regulated kinase
FAS	Fetal alcohol syndrome
GABA	Gamma amino butyric acid
GDNF	Glial-derived neurotrophic factor
GENSAT	Genetic nervous system Atlas
ICV	Intracerebroventricular
IGF	Insulin-like growth factor
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NGF	Nerve growth factor
NMDA	*N*-Methyl D-aspartate
NT	Neurotrophin
PACAP	Pituitary adenylate cyclase activating peptide

PI3K	Phosphatidyl inositol 3-OH-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PN	Postnatal
RACK	Receptor for activated C kinase
Trk	Tropomyosin-related protein kinase
VTA	Ventral tegmental area

1 Introduction

The majority of heavy drinkers who consume ethanol daily for years to decades show some form of brain pathology either as a result of alcohol-associated neurodegeneration or secondary to nutritional deficiencies, vascular damage, and hepatotoxicity (see Harper 2009; Zahr et al. 2011; Welch 2011; de la Monte et al. 2012 for excellent reviews). Ethanol is even more detrimental to the developing fetus, with Fetal Alcohol Syndrome (FAS) and Alcohol-Related Neurodevelopmental Disorders (ARND) occurring at a combined rate of <0.7 % in the United States to as high as 5 % in places like South Africa with high per capita alcohol consumption (de Sanctis et al. 2011; May et al. 2009). Chronic and developmental ethanol exposure clearly causes neurodegeneration and cognitive deficits; therefore, it seems intuitive that factors which mediate neuronal survival would be involved in ethanol-induced neurotoxicity. In models of ARND/FAS and in the adult, neurotrophins play a role in cell death, aberrant synaptogenesis, perturbed migration, tolerance, and repair but may also regulate the selective plasticity that occurs with addiction (reviewed by Ghitza et al. 2010; Castren 2004). Whether neurotrophin expression is the result of or induces subsequent changes in synaptic activity is not always clear, but neurotrophins are integral to many ethanol-sensitive processes.

Neurotrophins are a family of proteins initially characterized for the ability to provide trophic support to developing neurons but also regulate synaptic plasticity and survival in mature neurons (reviewed by Reichardt 2006). Nerve Growth Factor (NGF) was the first neurotrophic factor described which supported the growth of chick sensory ganglia neurons (Levi-Montalcini and Cohen 1960). This was followed by isolation and cloning of a family of proteins that includes Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and NT-4/5 in mammals and their respective Trk receptors (Leibrock et al. 1989; Klein et al. 1989; Lamballe et al. 1991; Maisonpierre et al. 1990b). Since these initial discoveries, many other factors have been isolated that are also trophic for neurons, but these factors do not contain regions of significant homology with the NGF family. Trophic factors explored in alcohol toxicology belonging to the latter group include Insulin-like Growth Factor 1 (IGF-1), Pituitary Adenylate Cyclase Activating Peptide (PACAP), and Glial-Derived Neurotrophic Factor (GDNF).

Classical neurotrophins of the NGF family are cysteine knot proteins that function as dimers. Neurotrophins are expressed as pro-proteins and cleaved to form active proteins, primarily through activity-dependent secretion and protease activation. Neurotrophins can be target-derived, transported trans-synaptically in a retrograde or anterograde direction, or signal through local mechanisms, depending on the brain region and neurotrophin (see Ginty and Segal 2002; Chowdary et al. 2012; Ascano et al. 2012 for reviews). Classical neurotrophins bind to Tropomyosin Related Kinase (Trk) receptors with picomolar affinity. TrkA is the full-length receptor for NGF, TrkB for BDNF and NT-4, and TrkC is the NT-3 receptor, although neurotrophins can bind other Trks with reduced affinity or differential coupling to signaling pathways (Schecterson and Bothwell 2010; Reichardt 2006). Trks also exist as splice variants that result in truncated receptors without signaling capacity, or with only the Shc binding domain (reviewed by Schecterson and Bothwell 2010). Trk receptors can also be transactivated by G protein–coupled receptors, adding further complexity to the signaling repertoire, potential for cell specific signals, and cross-regulation. Activation leads to dimerization and autophosphorylation of tyrosine residues in the full-length Trk receptors, creating docking sites for signaling intermediates (reviewed by Reichardt 2006). Modulation of signaling by ethanol exposure has been demonstrated for many of the major signaling pathways downstream of these trophic factors either individually, sequentially, or in parallel, including Extracellular signal Regulated Kinase (ERK), phosphatidylinositol 3-kinase (PI3K), phospholipase C, Protein Kinase C (PKC), small molecular weight GTPases, calcium, Nuclear Factor (NF) kB, and cyclic AMP (reviewed my Ron and Messing 2013). Neurotrophins are subject to activity-dependent, promoter-specific regulation and therefore regulated by synaptic activity, which is also directly regulated by ethanol exposure. In addition, novel and growth factor-convergent pathways have been identified in *Drosophila* that regulate ethanol sensitivity, tolerance, and toxicity that remain to be fully explored in mammalian systems (Devineni et al. 2011; Bhandari et al. 2009). Collectively, these data implicate neurotrophins as integral mediators that are regulated by, or regulate, nearly all neuronal processes, albeit with relative selectivity.

Classical neurotrophins also bind p75 with lower affinity, but pro-neurotrophins bind p75 with high affinity (reviewed by Koshimizu et al. 2009). p75 dimerized with sortilin family members to regulate Rho, NF-kB, and Jun N-terminal Kinase (JNK)-p53-Bax pathways, resulting in both survival and apoptosis (reviewed by Friedman 2010). Apoptosis through p75 is mediated by JNK while survival is signaled via NFkB in some cell types (Yeiser et al. 2004). p75 expression declines with age, indicating an active role in development. p75 can also dimerize with Trks to regulate activity, acting as dominant-negative receptors or enhancers of Trk signaling depending on the context, cell type, and neurotrophin concentration (reviewed by Schecterson and Bothwell 2010). For example, intracerebroventricular (ICV) injection of pro-NGF induces degeneration of the cholinergic system (Fortress et al. 2011) and mediates hippocampal growth cone collapse through p75-sortilin signaling to Trio-GEF-mediated Rac inhibition (Deinhardt et al. 2011).

Pro-BDNF and cleavage-resistant BDNF induce apoptosis and inhibit migration in cerebellar granule cells (Koshimizu et al. 2009, 2010). Cleavage-resistant pro-BDNF reduces cholinergic hippocampal innervation and hippocampal dendritic spines but does not appear to induce apoptosis in hippocampal neurons (Koshimizu et al. 2009, 2010). Pro-BDNF also mediates long-term depression (Woo et al. 2005). Thus, the maturation state of the neurotrophin, which is activity-regulated, may dramatically influence the receptor selectivity and the subsequent response of the cell.

This chapter will review the background on neurotrophin signaling, expression in animal models of ethanol exposure, and in patients with alcohol dependence. Each section will provide the necessary background information to evaluate the data. While not exhaustive in detail, these sections will provide references to the primary literature and more extensive reviews. Many studies differ in the presentation of dose or concentration and that should be considered. The LD50 for a single intraperitoneal dose of ethanol is ~3.5 g/kg for most mouse strains and range from less than 0.4 g/dL for some rat studies (Prado Carvalho and Izquierdo 1977) to as high as 7–9 g/kg in others (Material Safety Data Sheet). Concentrations are also given in percent (g/dL or mg/dL) or as millimoles per liter depending on the laboratory. Humans become unconscious at Blood Alcohol Concentrations (BACs) ~0.35 g/dL, and death is likely at concentrations ~100 mM or 0.5 g/dL. Therefore, LD50 should be considered in experimental situations where tolerance is not a factor. Besides the obvious complexities of dose, duration, tolerance, and withdrawal, several experimental caveats in inconsistencies must also be considered when reviewing 30 years of data speckled on a background of exponential scientific discovery and technological advances. These concerns will be addressed as they arise. Current data collectively suggest that work remains to be performed and replicated to gain a better understanding of how temporal changes in neurotrophin expression regulate, and are regulated by, physiological responses within the nervous system and how these influence neurotoxicity, plasticity, and repair after ethanol exposure.

2 Neurotrophin and their Receptors after Ethanol Exposure In Vivo: Animal Models

2.1 Nerve Growth Factor

2.1.1 NGF-TrkA Expression and Distribution

NGF was the first neurotrophin to be examined in models of alcohol-induced neurodegeneration and fetal alcohol syndrome. NGF is expressed in targets of sympathetic neurons and throughout the periphery, with high levels submaxillary gland, heart, prostate, stomach, endocrine system, skin, mast cells, intestine, spleen, lung, skin, and kidney (Korsching and Thoenen 1983; Heumann et al. 1984; Shelton and Reichchardt 1986). Within the CNS, NGF is trophic for Basal Forebrain Cholinergic Neurons (BFCN) where it upregulates choline acyltransferase (ChAT)

activity with an EC50 of ~3 ng/mL (Honegger and Lenoir 1982; Hefti et al. 1984; Mobley et al. 1986; Korsching et al. 1985). Functional studies and developmental expression indicate an essential role for NGF in hippocampal-dependent learning and expression of cholinergic markers in afferent structures (Conner et al. 2009; Conner and Varon 1997; Houeland et al. 2010). NGF also enhances communication between the nucleus basalis and the amygdala (Moises et al. 1995). Functional studies and the expression pattern implicate NGF as a candidate for modulation of ethanol-induced toxicity in the BFCN, hippocampus, and striatum, leading to cognitive deficits and addiction.

NGF mRNA expression (Fig. 1, Allen Institute) and GFP expression from the NGF promoter (Fig. 1, GENSAT) show the general distribution of NGF-producing cells in the adult mouse brain. Expression is sparse in comparison to BDNF shown in the adjacent panels. The highest level of NGF mRNA and protein is detected in the hippocampus within the mossy fiber terminal fields (hilus and stratum lucidum of CA3 and CA2) followed by cortex and olfactory bulbs (Fig. 1, Allen Institute and Gibbs et al. 1989; Maisonpierre et al. 1990a; Conner and Varon 1992; Altar et al. 1997; Conner and Varon 1997). Within the hippocampus, mRNA is localized primarily to interneurons of the hilus, but is also observed scattered in dentate granule cells, scattered interneurons within the CA fields, and interneurons in distal pyramidal cell synaptic fields after colchicine treatment (Ceccatelli et al. 1991). NGF mRNA is also detected in anterior olfactory nucleus, magnocellular preoptic nucleus, nucleus of the horizontal limb of the diagonal band, supramammillary nucleus, striatal interneurons, bed nucleus of the stria terminalis, ventral pallidum, mediodorsal thalamic nucleus, paraventricular hypothalamic nucleus, supraoptic nucleus, lateral and medial septum, substantia innominata, and nucleus basalis. Within the brainstem, NGF is localized to oculomotor, nucleus, facial nucleus, ambiguus nucleus, gigantocellular reticular nucleus, gigantocellular, reticular nucleus (alpha part), inferior olive, reticular nucleus, nucleus of the solitary tract, and vestibular nucleus (Maisonpierre et al. 1990a; Conner and Varon 1992; Altar et al. 1997).

The exact concentration of NGF (and all neurotrophins) in brain regions when measured by ELISA is variable due to extraction procedures, buffer composition, pH, fraction assayed, and the presence or absence of tissue homogenates in the standard curve (see Larkfors et al. 1987; Hoener et al. 1996; Fawcett et al. 1999; Zhang et al. 2000; Soderstom and Ebendal 1995; Whittemore et al. 1986). Calculated value from the studies cited above estimate NGF protein levels at (ng/g wet weight): hippocampus 1.34–6, cortex 0.57–3.5, olfactory bulb 0.85–5.3, basal forebrain 0.672–4.77, cerebellum 0.42–1.86 and striatum 0.16–1.0. Protein levels increase during development in the CNS with a peak that is approximately twice the adult level at postnatal day (PN)14 in cortex, PN14-21 in the striatum and BFCN, and PN7 in the cerebellum (Whittemore et al. 1986; Das et al. 2001; Mobley et al. 1986).

NGF is notoriously sensitive to fixation, pH, and post-fixation times; therefore, many studies purporting to measure NGF by immunohistochemistry may not be measuring NGF. Earlier studies discussed above are replicated in the mouse

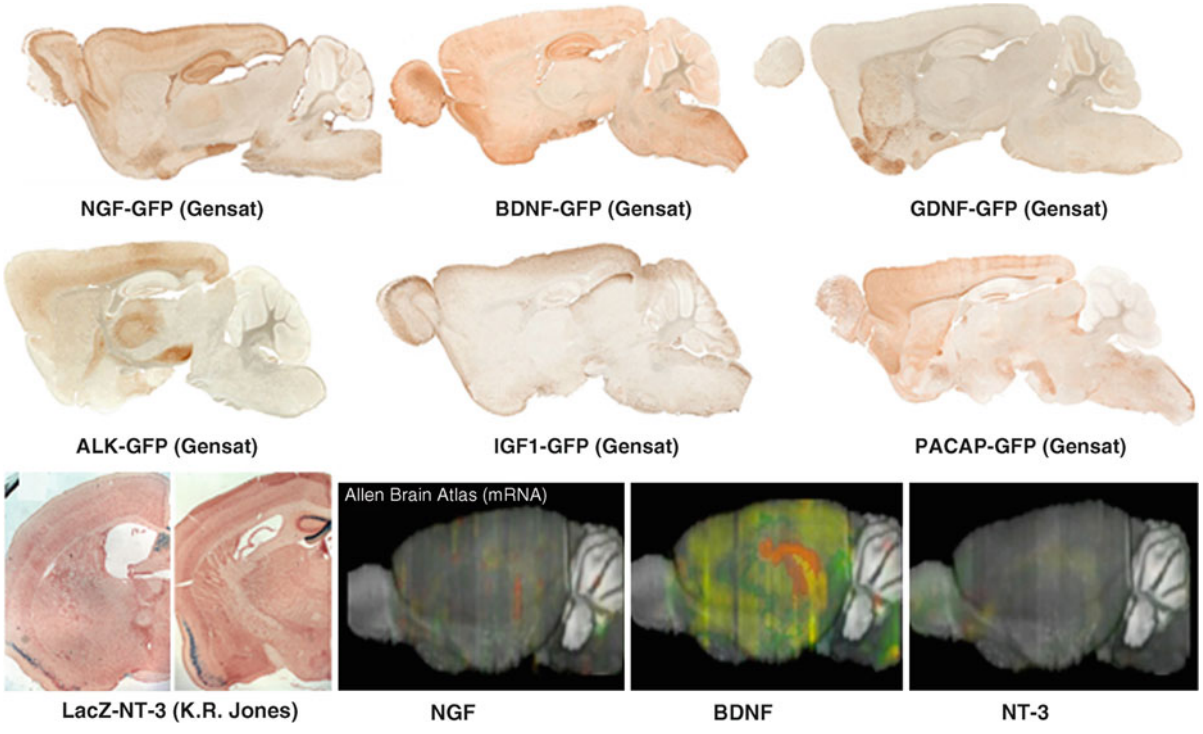

Fig. 1 Neurotrophic factor localization in the mouse brain using promoter-driven GFP expression and in situ hybridization. Immunohistochemistry for GFP in sagittal sections is shown for each neurotrophic factor to show the distribution of cells that produce the factors within the brain. Lac-Z expression from the NT-3 promoter in an adult mouse is shown in the *bottom left*. Rendered images from the Allen Brain Atlas mRNA series are shown for NGF, BDNF, and NT-3 in the *bottom row*. For more detail and immunohistochemical detection of each neurotrophin, see citations discussed in the text, GENSAT (www.gensat.org), and The Allen Brain Atlas (www.brain-map.org/) (Lac-Z images were generously provided by Dr. Kevin R. Jones and were generated as described in Vigers et al. [2000])

expressing GFP from the NGF promoter produced by GENSAT (Fig. 1). NGF immunoreactivity is particularly strong in hippocampal mossy fibers in mature and developing animals with transient production in other dentate lamina during development (Conner et al. 1992; Conner and Varon 1992, 1997). Cells in the stratum radiatum and stratum oriens contain NGF, while a few larger interneurons in the pyramidal cell layer contain NGF immunoreactivity (Conner and Varon 1992; Rocamora et al. 1996; Pascual et al. 1998). Cortical neurons immunoreactive for NGF were generally small (<12 um), but occasional NGF+ cells could be observed that were 13–17 μm in diameter, consistent with NGF expression primarily in interneurons (Conner and Varon 1992; Conner et al. 1992; Rocamora et al. 1996).

Regions of neurotrophin expression detected by mRNA frequently do not overlap with the protein distribution measured by immunohistochemistry. This is because NGF is produced in the target region or by local interneurons and retrogradely transported to the cell bodies of the "consumer" neurons (Seiler and Schwab 1984; Conner et al. 1992; Conner and Varon 1992). Colchicine treatment increases somatic immunoreactivity, allowing refined localization of NGF by allowing accumulation of protein in the cells that produce it and blocking retrograde transport to cells that stain for NGF because of uptake from postsynaptic sources. Under these conditions, NGF-expressing cells were detected in the olfactory bulb, tenia tecta, striatum, cortex, septum, BFCN, bed nucleus of the stria terminalis, diagonal band magnocellular preoptic region, ventral pallidum, and substantia innominata. Cholinergic cells with previous immunoreactivity in the septum and BFCN disappeared, thereby segregating the NGF "consumers" from "producers" (Conner and Varon 1992). Thus, many forebrain neurons will contain NGF protein but do not express NGF mRNA, instead obtaining NGF from cortical, hippocampal, and local interneurons (Conner et al. 1992; Conner and Varon 1992; Lauterborn et al. 1995; Whittemore et al. 1986). Similarly, much of the septal NGF is the result of retrograde transport from the hippocampus where it is expressed by cells in the target region for septohippocampal afferents (Conner and Varon 1992; Seiler and Schwab 1984). GFP expression from the NGF promoter also found NGF expression by Fananas astrocytes in the cerebellum and by cerebellar interneurons (Kawaja et al. 2011; GENSAT Fig. 1).

TrkA is widely expressed within the nervous system (see Sobreviela et al. 1994; Altar et al. 1991; Cohen-Cory et al. 1989 for detailed anatomy), including neurons of the striatum, ventral premammillary nucleus, mesencephalic trigeminal nucleus, vestibulocochlear ganglion, prepositus hypoglossal nucleus, raphe nucleus, nucleus ambiguus, glomeruli of the olfactory bulb, area postrema, and nucleus tractus solitarius. TrkA is not present in hippocampal pyramidal cells but is expressed by hippocampal interneurons. The suprachiasmic nucleus, olivary pretectal nucleus, and spinal trigeminal nuclei only contain TrkA immunoreactive fibers. TrkA immunoreactivity is also detected in the leptomeninges, ventricles, and ependymal cells (Gibbs et al. 1989). Iodinated NGF binding is highest in the interpeduncular nucleus and spinal trigeminal tract followed by striatum (higher in lateral striatum), amygdala, diagonal band, nucleus accumbens (NAc), olfactory tubercle, cerebellum, hippocampus, cortex, and subiculum. p75 is notably absent from striatal

cholinergic neurons that express TrkA (Sobreviela et al. 1994). Conversely, TrkA is not generally detected in mature cerebellar Purkinje cells that express p75 (Sobreviela et al. 1994; Larkfors et al. 1987, 1996). However, GFP expressed from the TrkA promoter does label a subset of Purkinje cells developmentally, suggesting that some transiently express TrkA. Interestingly, p75 regulates autophagy in Purkinje cells (Florez-McClure et al. 2004). NGF is also trophic for cholinergic interneurons of the striatal matrix in vivo (Van Vulpen and Van Der Kooy 1999) and in vitro (Studer et al. 1994) but has no effect on the survival of striatal medium spiny neuron (Ventimiglia et al. 1995), Purkinje cells (Larkfors et al. 1996), or hippocampal pyramidal cells (Ip et al. 1993).

Genetic deletion of NGF reduces acetylcholine esterase, causes a loss of sympathetic preganglionic neurons, reduces cholinergic septal and striatal neurons, leads to atrophy of BFCNs, and reduces hippocampal innervation by cholinergic afferents (Smeyne et al. 1994; Sanchez-Ortiz et al. 2012), while overexpression of NGF increases the size of septal neurons (Kawaja et al. 1998) and increases cholinergic neuron number and size without altering the innervation of the hippocampus (Fagan et al. 1997; Kawaja et al. 1998). Trk A knockout mice die by 1 month of age, exhibiting severe sensory neuropathies with smaller trigeminal, sympathetic, and dorsal root ganglia (Smeyne et al. 1994).

2.1.2 Ethanol and NGF Expression in Animal Models

NGF levels are sensitive to dose, duration, and withdrawal state, suggesting a dynamic role for NGF (and all neurotrophic factors) in intoxication, tolerance, and toxicity. Ethanol administered acutely via liquid diet causes an increase in NGF and TrkA protein and mRNA in the cerebellum (Wang et al. 2010). Chronic exposure via drinking water also produced a transient increase in NGF in the hippocampus, septum, striatum, cortex, BFCN, and olfactory bulbs (Gericke et al. 2006). After 9 months of exposure, there was a slight reduction in cortical NGF, but no differences in the other regions examined. Shorter exposures (2–4 weeks) of ethanol increased NGF in hippocampus and cortex with a return to control values after 3 months of ethanol exposure (Nakano et al. 1996). Chronic intermittent exposure produced an increase in hippocampal NGF protein levels (Miller 2004), but, in contrast to Gericke and colleagues, chronic exposure for 8 or 24 weeks reduced NGF in most cortical regions cortex and basal forebrain at most time points (Miller and Mooney 2004). Chronic ethanol exposure via liquid diet also reduced TrkA mRNA in the basal forebrain (Miller et al. 2002) and NGF can induce TrkA expression (Holtzman et al. 1992), suggesting failure of NGF to signal with chronic exposure. Combined with reduced basal forebrain TrkA (Miller et al. 2002), this increase in cortical and hippocampal NGF may reflect failure of NGF to transport or a neuroprotective induction that eventually fails with prolonged exposure. In contrast to chronic forced exposure, no differences in striatal NGF levels were observed by McGough and colleagues (2004) after 4 weeks of drinking using the 2 bottle choice model, suggesting some selectivity, depending on the model. The reason for these differences is unclear but may be the result of differential neurotoxicity, the choice to drink, or other environmental factors.

Bruns and Miller (2007) also observed an increase in NGF in the somatosensory cortex in layer V pyramidal cells, not interneurons, with repeated episodic exposure that correlated with the duration of exposure. NGF is generally not generally observed in pyramidal cells (Conner et al. 1992; Conner and Varon 1992; in contrast, see Pitts and Miller 1995; Pitts and Miller 2000), and only pyramidal cells were shown in the micrographs presented by Bruns and Miller; therefore, it is uncertain whether it is ethanol-specific ectopic expression, different pools of NGF, cellular uptake, or resulting from fixation procedures that were significantly different from Conner and colleagues (See Conner 2001 for detailed methods).

While the induction of NGF (i.e., Miller 2004; Gericke et al. 2006; Wang et al. 2010) may correlate with protective capacity and plasticity at early time points, many reports agree that long-term exposure to ethanol decreases NGF levels in the mature rodent hippocampus and cortex (De Simone and Aloe 1993; Aloe et al. 1993; Miller and Mooney 2004; Gericke et al. 2006). Furthermore, reduced hippocampal NGF after prolonged ethanol exposure is associated with atrophy cholinergic cells in the medial septum; similarly reduced cortical levels are associated with atrophy of septal neurons (De Simone and Aloe 1993; Aloe et al. 1993; Arendt et al. 1988; Cadete-Leite et al. 2003; Ehrlich et al. 2012). This atrophy and reduced NGF are accompanied by reduced ChAT activity in the hippocampus, septum, and striatum, which can be reversed by administered of NGF via osmotic pump (Cadete-Leite et al. 2003).

NGF-sensitive cells in the developing BFCNs and striatum show transient sensitivity to ethanol that parallel observations in the adult with variable resiliency. Initial studies used chick embryos to show that ethanol was toxic to cholinergic neurons, which was reversed by NGF, and established a window of vulnerability for these neurons (Rahman et al. 1993; Brodie and Vernadakis 1992). A similar window may exist in rodents; a single binge exposure on E15 resulted in a transient increase in hippocampal NGF at PN15 followed by a decline below control values in adulthood and parallel changes in ChAT (Angelucci et al. 1997, 1999).

Rats exposed prenatally to ethanol throughout gestation also have a transient reduction in ChAT activity in the BFCN, striatum, cortex, hippocampus, and septum neonatally (Swanson et al. 1995, 1996; De Simone and Aloe 1993; Fiore et al. 2009a, b). Reversal was associated with an increase in hippocampal NGF (transiently at PN7 and robustly at PN21) and repair in some studies (Heaton et al. 1996). In contrast, others (De Simone and Aloe 1993; Tsuji et al. 2008; Fattori et al. 2008) did not detect any differences in NGF in cortex with prenatal or postnatal exposure but reported a transient decrease in NGF in the hippocampus at PN7 that correlated with reduced cholinergic activity. Similarly, constant gestational exposure to ethanol (but not wine) reduced hippocampal and cortical NGF (Fiore et al. 2009a, b). This was correlated with a reduction in cholinergic neurons in the septum. The survival of cholinergic neurons and NGF induction differs with exposure paradigms, but each outcome is associated changes in NGF levels correlated with cholinergic neuron activity. Whether repair succeeds or fails may depend on the dose and duration of exposure, with binge exposure at a defined window being more toxic to developing BFCNs. As with the pattern in the adult where the ability to

upregulate NGF with intermittent exposure may be protective, variability in these data may be the result of the populations that survive the insult and their ability to produce NGF.

NGF levels are acutely increased in the cerebellum in mature mice by ethanol, suggesting a role in adaptation or repair (Wang et al. 2010), but NGF-ethanol interactions have primarily been addressed in the developing cerebellum. Cerebellar Purkinje cells are particularly vulnerable to ethanol toxicity during the first postnatal week but resistant to ethanol-induced cell death during the second postnatal week (West 1993), a period that coincides with the rise to the peak of developmental NGF expression (Das et al. 2001; Maisonpierre et al. 1990a). High dose ethanol (6.6 g/kg/day, peak BAC 336 mg/dL, cyclic exposure) PN4-10 via artificial rearing did not change NGF levels as detected by ELISA in neonatal cerebellar homogenates but reduced both p75 and TrkA in Purkinje cell dendrites and in cerebellar homogenates (Dohrman et al. 1997; also see Moore et al. 2004a). When this window was further subdivided, Heaton and colleagues (2003) found a transient reduction in NGF only when animals were exposed to ethanol at PN4 (correlating with the developmental period when Purkinje cells are sensitive to ethanol) but an increase at PN7 immediately after exposure and a delayed decrease at 12 h. Surprisingly, exposure on PN7-8 did not change NGF levels, suggesting some form of compensation with prolonged exposure, as was noted in the cholinergic populations. (The levels reported in this study (2003c) are two orders of magnitude greater than previously reported by this lab (i.e., Heaton et al. 2000c) and higher than classical reports. It is possible that this is calculation error, but no correction has been issued.) Furthermore, NGF overexpression from the GFAP promoter rescued Purkinje cells from ethanol toxicity in the neonatal cerebellum without hypertrophy (Heaton et al. 2000b). Purkinje cell number was slightly reduced in the control transgenic mice, suggesting that NGF overexpression alone in a non-activity dependent way may reduce Purkinje cell viability since hypertrophy resulting in fewer neurons per area is excluded but this was not evaluated. NGF overexpression induces aberrant cholinergic sprouting in the cerebellum that might also contribute to the increased survival in this paradigm through cholinergic systems positively coupled to survival pathways and independent of direct NGF effects on Purkinje cells (Kawaja and Crutcher 1997). Furthermore, the locus of NGF action in these studies has not been adequately established and again may suggest robust, dynamic changes in interneurons (Kawaja et al. 2011) or cerebellar afferent fibers since which then modulate Purkinje cell activity. Purkinje cells have not traditionally shown TrkA-mediated survival (Sobreviela et al. 1994; Larkfors et al. 1996) but this does not exclude a role for p75.

Upregulation of NGF at earlier time points after withdrawal or from low levels of ethanol may represent an attempt to compensate for the effect of ethanol on synaptic transmission that fails as producer neurons degenerate. Alternatively, the NGF measured may represent increased pro-NGF, leading to neurodegeneration. In addition, the effect of ethanol on the NGF system specifically in hippocampal and cortical interneurons has not been selectively evaluated in this context, but collectively these data suggest that interneurons may be a central mediator of

ethanol effects on NGF-mediated processes, perhaps secondary to reduction in synaptic activity, or death, with prolonged ethanol exposure. As discussed above, neurotrophic activity cannot be examined independent of the chronic and pharmacodynamic effects of ethanol on neural activity and circuit function.

2.1.3 NGF and In Vitro Models of Ethanol Exposure

Cell culture models have provided insight into ethanol pharmacology and toxicology, but many of the in vitro studies discussed below require supraphysiological concentrations of ethanol to induce cell death. These concentrations are generally more than 100 mM or 0.4 g/dL. Many dose–response curves in the studies discussed below range from 0.4 to 6 g/dL and require high concentrations of ethanol to decrease neurite outgrowth and induce cell death. Ethanol impairs neuronal function in vivo at much lower concentrations; therefore, the events leading to cell death in vivo are possibly more subtle, linked to developmental events such as migration and synaptogenesis, may be distinct from the overt toxicity observed over 24–48 h in vitro, and take longer to develop. Therefore, cell death may not be the result of immediate toxicity but may occur secondary to more subtle impairment in the developmental program that leads to cell death at a later developmental stage as the result of aberrant synapse formation or impaired migration. This caveat applies to similar sections on direct toxicity throughout this chapter.

As the prototypic neurotrophin, NGF was the first to be examined for direct modulation of ethanol toxicity in vitro. Chick sympathetic ganglia developed fewer neurites in response to ethanol in combination with NGF in vitro (Dow and Riopelle 1985) and impaired development of NGF-supported structures in ovo that could be reversed by exogenous NGF (Brodie et al. 1991; Brodie and Vernadakis 1992; Rahman et al. 1993). Dow and Riopelle (1985) used ethanol at concentrations ranging from 0.01 to 0.25 g/dL and evaluated neurite formation as a measure of toxicity but found no evidence for cell death at these concentrations. Follow-up in vitro studies replicated the decrease in neurites but extended the concentrations to include doses up to 6 g/dL (Heaton et al. 1993). Ethanol (0.25 g/dL) combined with NGF caused a slight (∼20 %) increase in processes in this model but higher concentrations reduced process formation and concentrations above 5 g/dL were required to kill dorsal root ganglia neurons. Similarly, cell death in cultured hippocampal and septal neurons did not occur at concentrations below 2.4 and 1.8 g/dL, respectively (Heaton et al. 1994). NGF was protective against toxicity, even at these extreme concentrations. When cultures were prepared from embryos exposed to ethanol in vivo, they were resistant to cell death at 1.8 g/dL but not 2.4 g/dL (Heaton et al. 1995), suggesting that exposure to ethanol increased resistance to subsequent exposure. In cortical organotypic cultures, NGF has region-specific effects, with neuroprotection only evident within the lower cortical layer developmentally (Mooney and Miller 2007). NGF-mediated neuroprotection is more dramatic in dispersed cultures of cortical, hippocampal, and cerebellar granule neurons (Heaton et al. 2000a; Mitchell et al. 1998; Seabold et al. 1998; Luo et al. 1997; Bonthius et al. 2003), although pyramidal cells and cerebellar granule cells do not respond to NGF through TrkA (Ip et al. 1993; Collazo et al. 1992;

Segal et al. 1992; Barker-Gibb et al. 2001; Courtney et al. 1997; Nonomura et al. 1996; Larkfors et al. 1996). These effects may instead be mediated by p75 in granule cells (Courtney et al. 1997). Alternatively, this may also be due to the relatively high concentration of NGF used, which can nonspecifically activate other Trks. While many neurotrophins are capable of increasing survival in ethanol-exposed cultures, some may be increasing baseline survival without a specific protective effect from ethanol (Bonthius et al. 2003).

PC-12 cells, an immortalized pheochromocytoma cell line with sympathetic neuron-like properties, express TrkA and show enhanced neurite formation, increased microtubule content, and enhanced differentiation in response to 100 mM ethanol (~0.48 g/dL) and NGF (Wooten and Ewald 1991; Messing et al. 1991; Roivainen et al. 1993; Roivainen et al. 1995). Enhancement was not seen with forskolin and ethanol, but FGF-induced neurite outgrowth was enhanced (Messing et al. 1991). This was accompanied by an increase in Thy-1 expression and subsequently determined to be mediated by PKC- and ERK-dependent pathways (Roivainen et al. 1993, 1995). PKC epsilon, a calcium-independent PKC isoform, was shown to mediate this enhancement of ERK (Hundle et al. 1997), while PKC delta, not epsilon, is responsible for the increase in polymerized microtubule content (Reiter-Funk and Dohrman 2005). Interestingly, PKC epsilon can induce neurite formation independent of catalytic activity (Zeidman et al. 1999) and inactive kinase induces more neurites than wild type PKC epsilon (Ling et al. 2004). Cell cycle arrest and enhanced differentiation were also observed in PC-12 cells, as determined by an increase in neuron-specific enolase expression, a decrease in ^{3}H-thymidine incorporation, and increased acetylcholine esterase expression with 87 mM ethanol in combination with NGF (Wooten and Ewald 1991). NGF-differentiated PC-12 cells are less sensitive to ethanol-induced cell death in the absence of serum (Oberdoerster and Rabin 1999) and, when specifically induced to differentiate with NGF, became less sensitive to ethanol inhibition of calcium channels (Mullikin-Kilpatrick and Treistman 1995). Therefore, ethanol causes cell cycle arrest and cell death in mitotic PC-12 cells, but once induced to differentiate by NGF, the signaling to ERK is enhanced by ethanol and neurite formation is enhanced, potentially protecting the cell from toxicity but with a potentially detrimental outcome if this were to occur in differentiating neurons.

Recent work using explant cultures of the nucleus basalis suggests more subtle effects than cell death in cholinergic neurons in response to ethanol (Ehrlich et al. 2012). Ethanol reduced ChAT immunoreactivity in neonatal explants without causing cell death or inflammatory cytokine induction which was reversible upon withdrawal. NGF (10 ng/mL) reversed the 100 mM ethanol-induced reduction in ChAT but did not reverse the reduction observed with 50 mM ethanol. Induction of trophic factors with the lower concentration could occlude an NGF effect since NGF was used near the EC50 in these experiments. Perhaps the ability of the cells to produce their own trophic factors is impaired at 100 mM, unmasking the effect. Furthermore, Erlich and colleagues implicate the nitric oxide synthase-p38 MAPK pathway in the reduction in ChAT with both 50 and 100 mM ethanol. These are

intriguing findings and support a role for NGF in repair after prolonged (7 days) ethanol exposure. The time course of NGF inductions varies significantly between studies and duration of ethanol exposure, but collectively, these data suggest induction of NGF that may mitigate some of the toxic effects of ethanol. NGF can be trophic, but if it is induced without concurrent neuronal activity and not cleaved, then pro-NGF can signal cytoskeletal changes or apoptosis through p75. Replicative experiments could focus on determining levels of pro and mature NGF, take advantage of newer technologies to determine the contribution of ethanol-induced changes in NGF and NGF signaling in survival, adaptive plasticity, addiction, and apoptosis.

2.1.4 NGF in Patients with Alcohol Dependence

Genetic polymorphisms and haplotypes in neurotrophins and their receptors in psychiatric and neurodegenerative disorders is an area of active research. The role of neurotrophin polymorphisms in neuropsychiatric disorders is controversial, and many initial association studies were not replicated. Others have reported no association with the underlying disorder but instead indicate a role for genotype in response to treatment (see Table 1). A few definitive associations of neurotrophin mutations with overt neurological disorders have been made, while most associations are weak or only relevant in subsets of patients. This applies to BDNF and other neurotrophic factors as well. Mutations and polymorphisms in NGF or TrkA have been associated with cancer (Scaruffi et al. 1999; George et al. 1998), hypertension (Kapuscinski et al. 1996), Alzheimer's disease (Di Maria et al. 2012; Nagata et al. 2011), anxiety (Lang et al. 2008), white matter structure (Braskie et al. 2012) and congenital insensitivity to pain (Mardy et al. 2001); however, unlike BDNF, no genetic associations with NGF polymorphisms have been reported with alcohol dependence.

Neurotrophins can be measured in the periphery and may be proportional to central levels as well as a surrogate for sympathetic dysfunction and peripheral neuronal activity. Table 1 details measurements of plasma and serum neurotrophin levels in patients with alcohol dependence and during withdrawal. The majority of the reports detailed in Table 1 indicate that ethanol increases peripheral NGF, regardless of the source (plasma vs. serum), in patients still drinking and during early withdrawal (Aloe et al. 1996; Lee et al. 2009; Heberlein et al. 2008; Jockers-Scherubl et al. 2007). NGF levels were found to be an order of magnitude higher in patients that had previously suffered delirium tremens and were not correlated with Korsakoff's Syndrome (Jockers-Scherubl et al. 2007). NGF levels decline toward normal during prolonged withdrawal but remain elevated for as long as 8 months (Aloe et al. 1996; Heberlein et al. 2008; Jockers-Scherubl et al. 2007). Furthermore, downregulation of NGF toward control levels was associated with methylation of the NGF promoter (Heberlein et al. 2011). In contrast, Yoon and colleagues (2006) reported decreased NGF after only 3 months of withdrawal. Ethanol produced a more dramatic decrease in NGF in patients with a family history of alcohol dependence. The patient demographics may contribute to these disparate

Table 1 Measurements of blood neurotrophin levels in patients with alcohol dependence

Study	Serum/ Plasma	NGF	BDNF	GDNF	Kit
Lee et al. (2009)	P	112 pg/mL C	861 pg/mL C		DuoSet ELISA
		137 pg/mL AD[a]	3502 pg/mL AD[a]		R&D
Huang et al. (2008)	S		15.8 ng/mL C		EMax ELISA
			13.9 ng/mL AD		Promega
			15.4 ng/mL 7d WD		
Joe et al. (2007)	P		822 pg/mL C		DuoSet ELISA
			384 pg/mL AD		R&D
			+FH 247 pg/mL		
			−FH 583 pg/mL		
Yoon et al. (2006)	P	110 pg/mL C			DuoSet ELISA
		71.9 pg/mL AD,			R&D
		+FH 64.7			
		−FH 83.3			
		>3 mos WD			
Heberlein et al. (2008)	P	19.87 pg/mL C			
		61.77 pg/mL AD			
		81.8 pg/mL AD[a]			
		21.68 pg/mL 1d WD			
		42.73 pg/mL 7d WD			
Jockers-Scherubl et al. (2007)	S	42 pg/mL			
		401 AD no DT			
		3292 AD + DT			
		225 1d WD			
		108 8d WD			
		144 3 m WD			
		128 8 m WD			
Costa et al. (2011)	S		24.2 pg/mL C		EMax ELISA
			23 pg/mL AD		Promega
			27 pg/mL AD[a]		
			32 pg/mL Abstinent		
Umene-Nakano et al. (2009)	S	21 ng/mL C			EMax ELISA
		9 ng/mL D			Promega
		9.8 ng/mL D + AD			
Meng et al. (2011)	S				Chemi-kine
					Millipore

(*continued*)

Table 1 (continued)

Study	Serum/ Plasma	NGF	BDNF	GDNF	Kit
Zanardini et al. (2011)	S		S-4.08 ng/mL C		Quanti-kine R&D
	P		S-4.77 ng/mL AD		
			P-41.7 ng/mL C		
			P-35.97 ng/mL AD upon admission		
Heberlein et al. (2010) Assoc with thrombocyte count and GGT levels	S		535 pg/mL C	196 ng/mL C	DuoSet ELISA R&D
			629 pg/mL AD 1d WD	82.7 pg/mL AD 1d WD	
			548 pg/mL AD 7d WD	93.5 pg/mL AD 7d WD	
			614 pg/mL AD 14d WD	81.4 pg/mL AD 14d WD	
Huang et al. (2011)	S		14.8 ng/mL C		EMax ELISA Promega
			12.3 No DT		
			6.2 DT		
			13.4 No DT, 7d WD		
			8.9 DT, 7d WD		
Aloe et al. (1996)	P[b]	32 C			Sandwich ELISA
		37 pg/mL Acute 1 hr			
		284 pg/mL AD			
		320 pg/mL Late WD			
		239 pg/mL Early WD			
D'Sa (2012)	P		S-26.6 ng/mL C		DuoSet ELISA R&D
	S		S-35.3 ng/mL AD		
			P ~ 1.3 ng/mL C		
			P ~ 1.4 ng/mL AD		

AD alcohol dependent, *C* control, *D* depressed, *WD* withdrawal, *FH* family history, *d* day, *m* month, *S* serum, *P* plasma

[a]Still drinking

[b]Results indicate plasma but methods state "No heparin, EDTA, or protease inhibitors were used"

observations. Patients were European in the first three studies, while the patients were Asian in the Yoon study and the control population varied by 11 years in age. While peripheral sources, concurrent anticonvulsant therapy, and systemic inflammation contribute to serum and plasma NGF levels, these do not appear to be significant covariates when measuring NGF levels in alcohol-dependent patients, nor does the blood component assayed. The well-established association between

stress and peripheral NGF levels (reviewed by Berry et al. 2012) also provides an intriguing link to alcohol dependence and NGF induction. The relative consistency among these studies implies global effects involving elevated NGF in homeostasis, withdrawal hyperexcitability, and repair after prolonged alcohol exposure that parallel observations in rodent models.

2.2 BDNF

2.2.1 BDNF-TrkB Expression and Distribution

While BDNF was the second neurotrophin to be characterized, BDNF distribution and functions are more widespread than NGF. Figure 1 shows the wide distribution of BDNF promoter–driven GFP expression and mRNA in the mature mouse. BDNF expression is regulated by multiple promoters that are differentially methylated and transcribed developmentally, regionally, by activity, pathologically, and upon exposure to xenobiotics (Pruunsild et al. 2007; Aid et al. 2007; Timmusk et al. 1993). BDNF mRNA targeting and translation are also regulated by 5′ and 3′ UTR sequences (Tongiorgi et al. 2006). BNDF expression level coincides developmentally with synaptogenesis and begins weakly at E13 in the brain (reviewed by Bartkowska et al. 2010). Levels of BDNF remain low until the second postnatal week which coincides with a peak in apoptosis for many structures (Das et al. 2001; Maisonpierre et al. 1990a; Katoh-Semba et al. 1997). BDNF levels are highest in the hippocampus (5.4 ng/g wet weight) followed by hypothalamus (4.33). All other brain regions contain less than 1.5 ng/g wet weight.

BDNF is expressed at high levels by cells in the cortex and hippocampus but is notably absent in the striatum, NAc, and striatal-derived amygdaloid structures (see Maisonpierre et al. 1990a; Ernfors et al. 1990a, b; Conner et al. 1997; Altar et al. 1997 for detailed expression and anatomy). Within the hippocampus, CA3 pyramidal cells and granule cells show the highest mRNA expression with lower expression in CA1 while hilar polymorph cells produce moderate levels of BDNF. BDNF-immunoreactive fibers populate the hippocampal strata oriens, radiatum, lucidum, and lacunosum moleculare, avoiding the stratum pyramidale. High levels of BDNF mRNA are also detected in the thalamus while both immunoreactive fibers and cell bodies containing mRNA are detected in the cortex, septum, BFCN, and the lateral and basal nuclei of the amygdala (Altar et al. 1997; Conner et al. 1997). As with NGF, BDNF is often synthesized in one area and transported, frequently anterograde (Conner et al. 1997; Altar et al. 1997; Li et al. 2012), but also retrograde as is observed in cerebellar granule cells (Bhattacharyya et al. 1997). Notable in addiction research, the medial habenula, striatum, NAc, central amygdala, and bed nucleus of the stria terminalis do not contain significant amounts of BDNF mRNA detected by in situ hybridization or BDNF-immunoreactive cell bodies but contain BDNF-immunoreactive fibers and cells expressing TrkB, consistent with anterograde transport to these (and other) regions (Altar et al. 1997; Conner et al. 1997; Baquet et al. 2004; Li et al. 2012),

The BDNF receptor TrkB is a family of proteins with thirty-six potential splice variants (Luberg et al. 2010), three of which have been well-characterized. TrkB.FL refers to the full-length receptor with tyrosine kinase activity. TrkB-Shc is truncated but retains Shc binding and coupling to the ERK and PI3K pathways. The truncated TrkB.T1 lacks tyrosine kinase activity and was thought to serve as a "decoy" to sequester and inhibit BDNF signaling, but TrkB.T1 coupling to Rho also regulates cytoskeletal dynamics (reviewed by Fenner 2012). TrkB splice variants are also believed to differentially regulate signaling intermediates, with full-length TrkB coupling to transcription through CREB and truncated variants signaling to the cytoskeleton. Both full-length and TrkB.T1 receptors are expressed throughout the brain, but the full-length receptor is exclusively associated with neurons, while TrkB.T1 is expressed by neurons, glia, choroid plexus, and ependymal cells (Altar et al. 1994). Iodinated BDNF and NT-4/5 binding sites were widely distributed similar to in situ hybridization (Altar et al. 1994). The highest levels of TrkB are in hippocampus, cerebellum, and cortex. Diencephalic, hypothalamic, and midbrain monoaminergic neurons express TrkB, and BDNF increases neurotransmitter synthesis and survival in these cells (Altar et al. 1992, 1994; Madhav et al. 2001; Akbarian et al. 2002; Baquet et al. 2004). TrkB is expressed on the majority of mesencephalic dopaminergic neurons, while many of these neurons also produce BDNF (Li et al. 2012; Numan and Seroogy 1999).

Homozygous BDNF knockout mice are not viable and die as neonates. Heterozygous mice exhibit a motor/balance phenotype by 2 weeks of age due to loss of vestibular ganglia (Klein 1994; Ernfors et al. 1994a; Jones et al. 1994). TrkB knockout mice do not eat and also die at birth (Klein et al. 1992). Reduced BDNF impairs taste (Liebl et al. 1997), resulting in abnormal appetitive behaviors in BDNF haploinsufficient mice (Kernie et al. 2000; see Krimm 2007) and, while controlled for and not observed in all experimental cohorts (Hensler et al. 2003; McGough et al. 2004), could contribute to increased alcohol consumption by BDNF haploinsufficient mice in some substrains. Deletion of BDNF also reduces neuropeptide expression in cortical interneurons but does not decrease parvalbumin- and NPY-expressing interneuron numbers (Jones et al. 1994). Recent work has employed region-specific Cre-recombinase-mediated gene deletion strategies and drug-inducible promoters to circumvent the developmental lethality of the full knockout. Combined, both of these strategies validate that deletion of, or reduction in, BDNF impairs hippocampal function, decreases striatal neuron survival, decreases spine density, and induces hyperactivity and hyperphagia (Lyons et al. 1999; Kernie et al. 2000; Rios et al. 2001; Zorner et al. 2003; Gray et al. 2006; Gorski et al. 2003; Li et al. 2012; Baydyuk et al. 2011). Cortex-specific and nigra-cortex double deletion of BDNF causes degeneration of striatal medium spiny neurons due to the loss of anterograde trophic support (Baquet et al. 2004; Strand et al. 2007; Li et al. 2012). Selective deletion of TrkB from medium spiny neurons impairs motor function, reduces striatal volume, MSN size, spine density, and impairs MSN dendritic development that is accompanied by a reduction in dopaminergic innervation (Baydyuk et al. 2011; Li et al. 2012). Interestingly, a human mutation in the TrkB gene is associated with developmental delay and severe obesity, a phenotype quite similar to the deficient mice (Yeo et al. 2004).

2.2.2 BDNF and Ethanol in Animal Models

A detailed review of ethanol and BDNF interactions in addiction and anxiety was recently published (Davis 2008); therefore, this section will concentrate on toxicity and recent controversial reports. BDNF has been extensively studied in the mature brain after acute and chronic ethanol exposure, but the results have varied significantly, often with disparate results in the same species, with the same exposure paradigm and over the same time course. For example, a few studies report no change in hippocampal BDNF levels (Miller et al. 2002; Okamoto et al. 2006), while most find a decrease in hippocampal BDNF with chronic exposure (MacLennan et al. 1995; Miller and Mooney 2004; Tapia-Arancibia et al. 2001; Hauser et al. 2011; Rueda et al. 2012). Still others report a transient increase in hippocampal BDNF with shorter exposure models (Kulkarny et al. 2011; Kalev-Zylinska and During 2007; McGough et al. 2004) and others see increased BDNF during withdrawal (Tapia-Arancibia et al. 2001) or with chronic intermittent exposure (Miller 2004). Disparate results were observed in BDNF mRNA in the hippocampus with intraperitoneal injection, the simplest of exposure paradigms. McGough and colleagues (2004) found an increase at 30 min while Raivio and colleagues (2012) examined levels at 3 h, implicating time as a factor; however, McGough did not observe a change in BDNF mRNA at this time point but found a reduction at 24 and 48 h. Raivio and colleagues conversely did not observe any differences in hippocampal BDNF at 24 h. Much of the discordant data are clustered around the acute time course as most studies agree that doses and exposure times sufficient to cause degeneration decrease BDNF but periods of withdrawal with intermittent exposure may be associated with attempts at repair (Miller 2004). These disparate results are observed in the same brain region and over the same time course, but collectively suggest that BDNF induction eventually fails in the hippocampus with prolonged exposure while upregulation during withdrawal may contribute to excitotoxicity and withdrawal-associated seizures.

Observations are inconclusive in other regions as well. Both increased and decreased BDNF protein levels have been observed in the cortex after chronic ethanol exposure and vary with region and pattern of exposure (Pandey et al. 1999; Miller and Mooney 2004; Miller 2004; Rueda et al. 2012). BDNF protein and mRNA levels in the cerebellum are acutely reduced by ethanol (Wang et al. 2010). BDNF mRNA levels in amygdala and NAc were also acutely reduced in rats after a single intraperitoneal dose of 2.5 g/kg of ethanol (peak BAC 58 mM); levels remained reduced in the frontal cortex at 24 h but returned to control values in the amygdala and hippocampus. The acute phase of this response is consistent with a decrease in excitatory transmission. BDNF mRNA in the NAc was initially reduced but increased in the ventral tegmental area (VTA) at 3 h, rebounding to increase at 24 h in both structures (Raivio et al. 2012). Kerns and colleagues (2005) also observed an increase in BDNF in NAc by microarray with acute exposure that was mouse strain specific. The acute increase in the VTA could result from direct excitation, reduced inhibition, or accumulation of mRNA that fails to transport but suggest that the VTA may be a source for accumbal BDNF mRNA levels since cortical levels remained lower. Microarray analysis also identified BDNF as a gene

with reduced expression in the prefrontal cortex after chronic intermittent ethanol exposure (Melendez et al. 2012). Interestingly, the array analysis also detected increased Oprl1 and extensive modulation of mRNA species belonging to the actin dynamics and MAPK signaling nodes consistent with a pharmacodynamic process. Consistent with a decrease in cortical BDNF mRNA, Bosse and Mathews (2011) measured BDNF protein levels in the striatum after acute ethanol exposure in both wild type and BDNF haploinsufficient mice. Both exhibited a decrease in BDNF; furthermore, the deficient mice did not respond to ethanol with a robust increase in dopamine when measured by in vivo microdialysis.

This is in contrast to work from the Ron/Janak laboratory that did not detect changes in cortical or NAc BDNF with acute exposure or self-administration (Logrip et al. 2009; McGough et al. 2004). Hippocampal BDNF was also elevated by ethanol through a Receptor for Activated C Kinase (RACK1) dependent pathway after both acute and prolonged (4 weeks) ethanol exposure (McGough et al. 2004; Jeanblanc et al. 2009, 2006; He et al. 2010). The data contrast the observations discussed above in extended striatum, implicating an acute increase in dorsolateral striatal BDNF that is negatively correlated with ethanol consumption (McGough et al. 2004; Logrip et al. 2009; Jeanblanc et al. 2009). This is supported by the observation that BDNF haploinsufficient mice voluntarily consume more ethanol than wild type litter mates, but these cohorts do not exhibit generalized hyperphagia, as discussed above (Hensler et al. 2003; McGough et al. 2004). Furthermore, viral mediated expression of BDNF siRNA in the dorsolateral striatum clearly transduced medium spiny neurons identified with GFP and replicated the phenotype of increased drinking (Jeanblanc et al. 2009). RACK1 mediated the ethanol-induced BDNF expression since Tat-RACK1 administration increased BDNF, reduced ethanol intake through TrkB, and reversed the phenotype in haploinsufficient mice when administered in the dorsolateral striatum (McGough et al. 2004; Jeanblanc et al. 2006). BDNF induction by ethanol was also observed in vitro with immunofluorescence in striatal neurons (Logrip et al. 2008), neuroblastoma cells (He et al. 2010), and in hippocampal pyramidal cells (McGough et al. 2004; He et al. 2010), requiring RACK1 for gene expression in each cell type. RACK1 activation of BDNF transcription is purported to be mediated by cAMP (Yaka et al. 2003b, c; He et al. 2010; Neasta et al. 2012), 14-3-3 association (Neasta et al. 2012) and epigenetic modulation of BDNF promoter IV (He et al. 2010). Interestingly, cAMP signaling via PACAP also appears to use this pathway to induce BDNF (Yaka et al. 2003a, b). Cyclic AMP causes dissociation of RACK1 from the NMDA/Fyn complex, thereby increasing Fyn phosphorylation of the NMDA receptor and increased NMDA activity. Collectively, these data place cAMP upstream of many key events involved in adaptation to ethanol exposure but implicate RACK1 and not CREB as the key transacting factor in this pathway.

Similarly, BDNF induction was observed in MSNs of the central and medial amygdala by Pandey and colleagues, where interference with BDNF expression using antisense oligonucleotides increased anxiety-like behavior and ethanol consumption (Pandey et al. 2005). Furthermore, alcohol-preferring rats produced less central amygdala and BNST BDNF mRNA and protein and exhibited higher

anxiety-like behavior (Moonat et al. 2011; Prakash et al. 2008). In this case, CREB appears to be a key intermediate in the behavioral response but Pandey and colleagues place CREB and ERK downstream of BDNF induction. While Pandey's group did not detect strain differences in BDNF in the NAc, Yan and colleagues found a reduction in accumbal BDNF in the preferring strain (Yan et al. 2005). Together, these data implicate reduced BDNF in the basal ganglia in alcohol appetite, but the anatomical locus is not resolved. As with MSNs in the striatum, BDNF protein should be delivered to the central amygdala via afferents (Conner et al. 1997) but the source of the mRNA and efficacy of BDNF siRNA and antisense oligonucleotides were unequivocally shown to be locally mediated within medium spiny neurons in the dorsal striatum and striatal-like nuclei of the amygdala. This was not concluded by Riovio (2012) and colleagues whose data and discussion suggest afferent or rare local sources in the accumbens. These data are difficult to reconcile and will require further anatomical, pharmacological, and molecular investigations.

Variable results have also been reported in developing animals with BDNF expression, likely amplified by the low levels of BDNF in neonates and stress. Many fetal alcohol experiments focus on the developing cerebellum because the ontogeny of specific neuronal populations, their migration, synaptogenesis, and the factors modulating these processes have been well-characterized (reviewed by Sotelo 2004). The majority of studies examining BDNF levels, TrkB, or BDNF signaling have shown that ethanol inhibits the neurotrophic activity of BDNF in the cerebellum, and this may contribute to cerebellar apoptosis and impaired foliation. Ge and colleagues (2004) performed a detailed dose–response, time-course analysis and found a rapid decrease in BDNF and TrkB mRNA in the cerebellum after exposure on PN4 but not PN9, suggesting a role for BDNF in the selective vulnerability at this time point when levels are low. In a follow-up study (Light et al. 2002), TrkB was detected in cerebellar Purkinje cells during the vulnerable first postnatal week; ethanol decreased TrkB immunoreactivity as well as TrkB.T1 and TrkB mRNA in these experiments. Moore and colleagues found an increase in TrkB in the cerebellum of females exposed prenatally but no other alterations in cerebellar TrkB with prenatal or postnatal exposure (Moore et al. 2004a, b). Moreover, Heaton and colleagues did not observe significant regulation of BDNF protein levels in cerebellum with numerous exposure windows that span postnatal development but subsequently reported a transient increase in BDNF immediately after exposure on PN4 that normalized by 2 h (Heaton et al. 1999, 2003a). Exposure on PN7 increased BDNF levels at 2 h and decreased levels at 12 h in this study. Observations made in juvenile rats after a single binge of ethanol indicate no changes in cerebellar BDNF mRNA (Kulkarny et al. 2011), suggesting modulation may be more dynamic in younger animals.

Multiple lines of evidence support a role for BDNF expression and signaling in the cortex and hippocampus in the development of FASD, but the sequence and mechanisms have not been fully resolved. A few reports have suggested an increase in BDNF (and related TrkB agonist NT-4) immediately following exposure. Increased BDNF/NT-4 levels were measured in combined "cortex/striatum" at

PN1 after prenatal exposure and at PN10 after PN4 -10 (Heaton et al. 2000c). Surprisingly there was no significant developmental increase detected in BDNF between PN10 and PN21. By contrast, Heaton and colleagues (2003a) reported bidirectional changes in BDNF cortex after exposure on PN7 and increased BDNF on PN10 after neonatal exposure that resolved by PN21. It should be noted that the levels of BDNF measured in these assays were very low (50–80 pg/g wet weight) and may have been below linear detection with the dilutions given in the text (100-fold). Others have found that fetal exposure decreases BDNF in the cortex (Climent et al. 2002; Fattori et al. 2008; Feng et al. 2005; Caldwell et al. 2008). In addition, Caldwell and colleagues (2008) found decreased BDNF and specific decreases in transcripts III, IV, and VI in the medial frontal cortex and hippocampus.

Similar differences were observed in the hippocampus where Heaton and colleagues (2000c) find a transient increase in hippocampal BDNF at PN10 with neonatal exposure and no change with prenatal exposure. However, Fattori found a decrease in BDNF after PN5-8 exposure. Feng and colleagues (2005) and Caldwell colleagues (2008) also report a decrease in BDNF after gestational exposure. Miki et al. (2008) exposed animals from PN10-15 via vapor inhalation and also noted a transient increase in hippocampal BDNF mRNA from PN16-PN30 that varied in duration depending upon the control group comparison. These observations were less robust in older animals and complicated by differential BDNF expression between the maternally reared control group and the separation control group such that BDNF levels were reduced when compared to the separation control animals but unchanged relative to the maternally reared animals at 60 days of age (Miki et al. 2008). Similarly, separation of PN5 pups increased PKC activity in the hippocampus, which correlated with increased ERK signaling (Davis et al. 1999). These observations illustrate how stress significantly affects signaling in the developing brain and may be another variable contributing to the differential effects of ethanol on neurotrophin levels and signaling between laboratories and exposure paradigms. While it is difficult to draw firm conclusions from the current data, combined, these studies suggest that ethanol may acutely and transiently increase BDNF in some brain regions developmentally. Correlations with cell death suggest that the increase may reflect an attempt at repair, but may ultimately lead to a reduction in BDNF production in the mature structure if administered during a vulnerable window.

Ethanol also produces variable effects on TrkB that may be dependent not only on timing but also on gender (Moore et al. 2004a, b). However, in spite of this overwhelming confound, others have found that prenatal exposure increases TrkB/TrkB-T1 ratios as neonates (Climent et al. 2002). Baseline neurotrophin levels and BDNF levels are responsive to multiple environmental and experimental factors that can make accurate measurements difficult. More experiments examining acute effects on signaling during exposure and compensatory regulation over time and after in specific cell types are required. It is also unclear whether these changes result in a decrease in activity-dependent BDNF release or represent differences in synaptic complexity. Examination of BDNF signaling (discussed below) suggests that ethanol causes a decrease in the ability of TrkB to transduce signal and desensitize, which may lead to compensatory regulation of both BDNF and TrkB

in vivo. There is considerable data to indicate a role for aberrant BDNF modulation during post-exposure development, but this may be secondary to impaired synaptogenesis, activity, and neuronal apoptosis (Bhave et al. 1999; Feng et al. 2005; Miki et al. 2008; Caldwell et al. 2008; Climent et al. 2002).

2.2.3 BDNF and In Vitro Models of Ethanol Exposure

As with NGF in the previous section, BDNF diminishes ethanol-induced hippocampal and cerebellar granule cell death in vitro (Heaton et al. 2000b; Bonthius et al. 2003). The neuroprotective effects of NMDA in cerebellar granule neurons are also mediated by BDNF induction (Marini et al. 1998; Bhave et al. 1999) and likely involve PI3K since the survival-promoting effects of BDNF were blocked by PI3K inhibition (Bhave et al. 1999). Ethanol also decreased Akt phosphorylation in the developing, but not adult, brain (Chandler and Sutton 2005), implicating PI3K/Akt in the selective vulnerability of the developing brain to ethanol.

Neurotrophins also reverse many of the effects of ethanol on synaptic activity. Exogenous BDNF reverses ethanol attenuation of GABA-A responses in cerebellar granule cells (Ericson et al. 2003). Ethanol also reverses BDNF-mediated long-term plasticity at developing hippocampal CA3 GABAergic synapses through inhibition of voltage-gated calcium channels (Zucca and Valenzuela 2010). At excitatory synapses, ethanol blocks BDNF enhancement of NMDA (NR2B-containing), but not AMPA, responses in hippocampal neurons (Kolb et al. 2005). As a control, the authors show that NGF and NT3 do not alter synaptic transmission, indicating cell type selectivity in the ion channels modulated by specific neurotrophins as would be expected from the anatomical distribution discussed above. Kolb and colleagues further dissected the effect of ethanol on the channel from the BDNF enhancement by using ethanol concentrations that did not have an impact on NMDA signaling, indicating that the effect of the effect of ethanol is independent of NMDA receptor inhibition.

In contrast to the experiments discussed above where chronic ethanol enhanced NGF-stimulated ERK activity in PC-12 cells, acute and chronic ethanol administration decreases activation of ERK in primary neurons and in the rodent brain (Davis et al. 1999; Kalluri and Ticku 2002, 2003; Tsuji et al. 2003; Chandler and Sutton 2005; Han et al. 2006; Ohrtman et al. 2006; Fattori et al. 2008 however, see Chen and Charness 2012 and Acquaah-Mensah et al. 2001). Regulation of ERK by ethanol also contributes to reduction in hippocampal LTP (Roberto et al. 2003). Ethanol acutely reduces TrkB signaling to ERK in cerebellar granule cells (Li et al. 2004; Ohrtman et al. 2006), but does not change TrkB phosphorylation state (Li et al. 2004), indicating an intracellular site of action downstream of the receptor. Neonatal hippocampal slices stimulated with BDNF displayed reduced nuclear translocation of phosphorylated ERK in hippocampal pyramidal cells in CA1 (Davis et al. 1999). Inhibition of BDNF-stimulated ERK phosphorylation by ethanol in cerebellar granule cells is dependent upon BDNF concentration and not modified by NMDA receptor antagonists (Ohrtman et al. 2006). Low concentrations of ethanol inhibit ERK activation only when BDNF was present at concentrations in the rising portion of the high affinity concentration–response curve (<5 ng/mL), while high concentrations of ethanol also block the desensitization of the ERK response at longer times (30–60 min), and at high BDNF

concentrations (>10 ng/mL). Thus, there was no apparent inhibition with 50 ng/mL of BDNF after 20 min, but the peak was never reached in the ethanol-exposed cells while the control curve is declining (Ohrtman et al. 2006). This was also reported recently by Chen and Charness (2012) who did not observe ethanol inhibition of BDNF-stimulated ERK activity with 50 ng/mL of BDNF, but this study did not perform the same pharmacological analysis as Ohrtman and colleagues. Both PKA and PKC regulate ERK, and reduced ERK activity correlates inversely with PKA activity and positively with a concurrent decrease in calcium-sensitive protein kinase C activity in the developing hippocampus (Davis et al. 1999); therefore, acute inhibition of TrkB signaling to ERK may be independent of Trk activation and ultimately cross-regulated by other pathways with different concentrations of ethanol.

In addition to mediating survival, BDNF also regulates migration and synaptogenic processes. Mutations that either upregulate or downregulate signaling through growth factor pathways and their associated GTPases cause a myriad of neurodevelopmental disorders resulting from accelerated or decelerated development (Stornetta and Zhu 2011). Perturbation of signaling that results in subtle changes in the rate of migration and synaptogenesis might also contributes to the development of alcohol-related neurodevelopmental disorders without inducing overt cell death. Numerous reports indicate that concentrations of ethanol as low as 25 mM inhibit neuronal migration neurite outgrowth through the adhesion molecule L1 (Charness et al. 1994; Bearer et al. 1999), and inhibition of ERK and Src and implicated in inhibition of L1-mediated neurite formation by ethanol (Tang et al. 2006; Yearney et al. 2009). In contrast, a few studies report increased migration and increased neurite formation in response to ethanol, as discussed above, and increased basal spines on pyramidal cells treated with chronic intermittent ethanol (Kroener et al. 2012). Developmentally, Powrozek and Olson (2012) observed increased dendrite formation in cortical explant cultures treated with ethanol. Proliferating neuroepithelial precursors exposed to ethanol display a differentiation-associated increase in migration (Camarillo and Miranda 2008) and ethanol increased the rate of axon formation in hippocampal pyramidal cells (Lindsley et al. 2011). This overall increase in axon growth rate was associated with ethanol inhibition of BDNF-stimulated Rac1/Cdc42 activation and increased Rho activity in hippocampal axonal growth cones, leading to increased growth cone surface area (Lindsley et al. 2011). Although ethanol increased the surface area of growth cones, the levels of active Rho GTPases in axonal growth cones were not affected in the absence of exogenous BDNF. These observations are in contrast to a recent study from Chen and Charness (2012), detailing reduced axon growth that was not mediated by BDNF and was dependent on Src, albeit with different substrates. We have also observed an increase in BDNF-stimulated migration/dendrite formation in cerebellar granule cells using a Boyden chamber assay (Hassoun et al. 2007a; See Hassoun et al. 2007b for detailed methods). As discussed above, ethanol also enhances differentiation in some cells, therefore cell cycle arrest, possible through ERK inhibition in cycling cells, and premature differentiation would increase the pool of migration-competent precursors if administered during the correct developmental window. This does not necessarily have to increase the rate

of migration, which has been shown to be reduced in the cerebellum after ethanol exposure (Jiang et al. 2008), but may simply increase the number of cells that migrate toward BDNF.

Oxidative stress, also known to modulate neurotrophin signaling, has recently been implicated in the etiology of some forms of autism (Rossignol and Frye 2012; Sajdel-Sulkowska et al. 2011) as well as the development of FAS/ARND (Brocardo et al. 2011). A target that has been suggested to mediate the toxic effects of ethanol is cJun N-terminal Kinase (JNK), a member of the mitogen-activated protein kinase family, which is induced by ethanol, genotoxicity, and oxidative stress (reviewed by Weston and Davis 2007). p75 also activates the JNK pathway (Koshimizu et al. 2010). JNK, as the name implies, phosphorylates c-Jun, a member of the AP-1 (Fos/Jun) transcription factor complex. "JNK" represents 3 families and as many as 16 splice variant isozymes that do not always correlate with molecular weight but is generally divided into 3 molecular weight categories of 58, 54, and 46 kDa. Suppression of Akt activates JNK (Shimoke et al. 1999), but apoptosis in cerebellar granule cells induced by growth factor withdrawal, glutamate, and beta amyloid is not mediated by JNK (Gunn-Moore and Tavare 1998). Some reports suggest that JNK with an apparent molecular weight near 60 kDa is involved in ethanol-induced apoptosis and ethanol-induced oxidative stress, but only one isoform was observed by western blot (Heaton et al. 2003a, 2012). Han and colleagues (2006) observed ethanol-induced increases in p46 and p54 phosphorylation that are associated with apoptosis in PN7 rat pups. However, when phosphorylated cJun is measured as a surrogate for JNK activity after ethanol exposure, there is no overlap between cells undergoing apoptosis and phospho-cJun in immunostained brain sections (Young et al. 2008). Moreover, JNK activation induces genes associated with protection in *Drosophila* in response to oxidative stress (Wang et al. 2003) and mutations that enhance JNK signaling in Drosophila can extend lifespan (Karpac and Jasper 2009). BDNF stimulation of AP1 DNA binding activity is also reduced by ethanol in cerebellar granule cells (Li et al. 2004). The effects of JNK activation may therefore be isozyme or splice variant, specific as these data suggest reduced genomic signaling through Fos/Jun after ethanol in cerebellar granule cells and a non-genomic, possibly protective, isoform-specific, role for JNK in ethanol toxicity which has not been explored.

The JNK pathway stimulates both dendrite and axon formation through phosphorylation of proteins such as MARKS and doublecortin that are associated with cytoskeletal dynamics (Oliva et al. 2006; Bjorkblom et al. 2012; Jin et al. 2010). JNK1 is associated with microtubules where it regulates axodendritic length (Tararuk et al. 2006), and genetic deletion of JNK or intermediates in the JNK pathway leads to axonal growth defects (Chang et al. 2003; Koushika 2008). Immunohistochemical staining for phospho-JNK in the neonatal mouse brain labels primarily developing axon pathways and not apoptotic cell populations (Davis, unpublished observations). A role for JNK regulating migration and axon outgrowth in response to ethanol is further supported by the observations that ethanol increases integrin and NCAM levels (Vangipuram et al. 2008; Miller et al. 2006), increases laminins (Vangipuram et al. 2008), increases Fyn activity (Yaka et al. 2003b), increases PKC, and increases FAK phosphorylation in cerebellar granule cells (Sivaswamy et al. 2010), which all regulate

cytoskeletal dynamics (Becchetti and Arcangeli 2010). JNK may therefore be an overlooked contributor to ethanol effects on the neuronal cytoskeleton and may serve protective as well as apoptotic functions.

2.2.4 BDNF in Patients with Alcohol Dependence

Variability in peripheral BDNF levels in alcohol-dependent patients is more extreme than NGF, making it difficult to deduce trends from the current data. The levels of BDNF reported in Table 1 are variable, often with standard deviations greater than the mean. Stress, depression, hematologic parameters, xenobiotics, age, and gender are all known to influence peripheral BDNF levels (reviewed by Autry and Monteggia 2012; Lommatzsch et al. 2005). BDNF levels detailed in Table 1 do not correlate with the source (plasma vs. serum), method used, or duration of abstinence. The source of the variability could be innate, due to simultaneous use of anticonvulsants during withdrawal, or due to sample preparation. Many experiments used serum, which includes BDNF released by thrombocytes, and is orders of magnitude higher than the plasma concentration (Lommatzsch et al. 2005). Two studies state different sources in the methods compared to the results section, although the levels reported suggest the source (ng vs. pg concentrations). In other studies, it is not clear that the centrifugation steps are sufficient to remove the thrombocytes, while others use sedimentation forces likely to rupture the cells. Zanardini and colleagues (2011) and D'Sa and colleagues (2012) directly compared serum and plasma BDNF. The first study reported a small increase in both components, while the second study only detected an increase in BDNF in serum in alcohol-dependent patients. Heberlein and colleagues (2010) also addressed this confound by simultaneously measuring thrombocyte activity and serum BDNF levels, observing a positive correlation between serum BDNF levels and both gamma glutamyl transferase levels and thrombocyte function. In addition, Costa and colleagues (2011) found a nonsignificant but suggestive association of BDNF levels with platelet count. Alcohol dependence and withdrawal are known to alter thrombocyte levels and coagulation cascades with robust rebound upon withdrawal (Schmitt et al. 1999; Chanarin 1982). BDNF is also produced and released by the vascular endothelium (Nakahashi et al. 2000), further complicating any correlation between central function and peripheral levels in patients with underlying cardiovascular disease.

Peripheral levels may provide valuable information since the production and release mechanisms regulating plasma and serum levels are likely to be similar to mechanisms governing central production and release, even if they are not the same pools of BDNF. Interestingly, this was predicted by Chinarin in 1982: "After platelet aggregation the platelets contract and squeeze various metabolites out of the platelet; this is called the 'release reaction'. This phase is also curtailed or even absent in platelets from alcoholics". BDNF polymorphisms also have the potential to regulate peripheral levels, but these reports are similarly variable and primarily examine serum BDNF. The Met allele of the Val66Met BDNF polymorphism decreases neuronal BDNF secretion (Egan et al. 2003; Chen et al. 2004, 2006). Some evidence supports an association between the homozygosity for the Met or

Val allele with lower or higher BDNF levels, respectively (Ozan et al. 2010; Cirulli et al. 2011). In contrast, no association between genotype and serum levels were found in the majority of patient populations examined thus far (Yoshimura et al. 2011; Trajkovska et al. 2007; Terracciano et al. 2011) and, paradoxically, patients with the Met allele were even found to have higher serum BDNF concentrations (Lang et al. 2009). Thus, factors other than genotype may be responsible for the majority of the variability in serum and plasma BDNF.

BDNF is highly polymorphic and linkage analysis identified an alcoholism susceptibility gene that mapped near the BDNF locus, 11p13 (Uhl et al. 2001). The human BDNF gene is encoded by 11 exons and 9 promoters (Pruunsild et al. 2007) and is polymorphic between different human populations, further complicating association analysis (Petryshen et al. 2010). Three polymorphisms have been examined in alcohol-dependent patients; these polymorphisms change BDNF secretion and activity-dependent processing (Egan et al. 2003; Chen et al. 2004, 2006). The first is a missense change (G196A, rs6265) that results in a valine to methionine substitution (Val66Met). The Met allele shows impaired trafficking and activity-dependent secretion, and humans homozygous for the Met allele have reduced hippocampal and amygdalar volume, increased anxiety, increased heart rate, and poor working memory (Egan et al. 2003; Hariri et al. 2003). Humans homozygous for the Met allele have recently been reported to have higher anxiety, increased cortisol, and consume more alcohol (Colzato et al. 2011). Abstinent Met/Val and Val/Val patients differ in their rate and locus of recovery measured by MRI, with Val/Val homozygotes showing more gray matter recovery while heterozygotes had more recovery in white matter measures (Mon et al. 2012). Transgenic mice carrying the human Met allele and their human counterparts were also impaired in models of extinction learning, with human Met/Met homozygotes demonstrating impaired fronto-amygdaloid activity by functional magnetic resonance imaging (Soliman et al. 2010). A second polymorphism (C270T) is present in the 5′ noncoding region that can regulate transcription or translation. The third genotype examined is a G712A polymorphism that has not been extensively investigated molecularly but may be associated with Parkinson's Disease (Chen et al. 2011). Initial studies suggested BDNF polymorphisms were associated with psychiatric disorders; however, subsequent studies and meta-analysis have failed to support associations for most major neuropsychiatric disorders, while studies contain too few patients to asses validity in less common disorders at this point (reviewed by Hong et al. 2011).

Anxiety is a precipitating factor for alcohol abuse. Association between the Met allele and higher anxiety-like behavior has been observed in both humans and transgenic mice expressing the human Met allele (Jiang et al. 2005; Montag et al. 2010; Chen et al. 2006). BDNF polymorphisms have been examined by several groups and the associations with alcohol dependence have been weak and variable in spite of promising initial observations. Matsushita et al. 2004 found that G196A (Val66Met) BDNF polymorphism frequency did not differ significantly from controls but alcohol-dependent patients with violent tendencies and a history of delirium tremens had an increased frequency of Met/Met genotype. Antisocial

alcohol behavior and alcohol dependence may also segregate with a TrkB variant (Xu et al. 2007). The Met allele was also associated with earlier disease onset in this study. Met carriers were subsequently shown to consume more alcohol than Val66Val or Val66Met patients which segregated with alcohol use disorders (Shin et al. 2010; Colzato et al. 2011). Reduction of BDNF is associated with aggression in mice (Chen et al. 2006), but the genetic association between aggression, Met/Met, and alcoholism was not observed in the majority of replicate analyses (Tsai et al. 2005; Muschler et al. 2011; Wojnar et al. 2009; Grzywacz et al. 2010). In a subgroup analysis, the Met allele was overrepresented in alcohol-dependent patients with depression compared to controls or dependent patients without depression (Su et al. 2011). While no association was found with dependence, Wojnar and colleagues (2009) found an association between the Val66Val genotype and relapse that was greater when restricted to analysis of those with a positive family history of alcohol use disorders. In support of this phenotypic association, the Val/Val is also associated with impulsivity (Gatt et al. 2009), which can contribute to relapse. A modest association between SNP G712A and substance dependence was also described but did not specifically examine the alcohol-dependent subgroup (Zhang et al. 2006). These data do not suggest a strong association between the BDNF polymprphisms and alcohol dependence but may identify a subgroup of patients and indicate response to treatment. Interestingly, over 60 polymorphisms in the BDNF gene have been identified to date, so lack of association at two loci does not exclude a role for BDNF genotype as a modifying factor in alcohol abuse, dependence, and toxicity.

2.3 NT-3

2.3.1 NT-3-TrkC Expression and Distribution

NT-3 is widely expressed in the periphery, with high levels in the kidney, spleen, heart, and muscle (Maisonpierre et al. 1990a; Ernfors et al. 1990b). As with NGF and BDNF, NT-3 supports survival and neurite outgrowth from peripheral ganglia (Maisonpierre et al. 1990a; Ernfors et al. 1990b). With the exception of hippocampal dentate granule cells, NT-3 mRNA is not highly expressed in the mature CNS (Fig. 1, Allen Institute and LazC expression from the NT-3 promoter generously provided by Dr. Kevin Jones at the University of Colorado). NT-3 is present at low levels in mature animals in the indusium griseum, tenia tecta, olfactory bulb, substantia nigra, medial locus coeruleus, and scattered paraventricular (IV) brain stem nuclei (Ernfors et al. 1990b; Vigers et al. 2000). Other structures such as the retrosplenial and visual cortices with low NT-3 expression and a developmental peak were also identified in a mouse strain expressing LacZ from the NT-3 promoter (Vigers et al. 2000). LacZ expression is more sensitive than in situ hybridization, but this essentially replicated the earlier mRNA studies with respect to relative expression levels, including lack of NT-3 expression by cells in striatum and globus pallidus (Maisonpierre et al. 1990b; Friedman et al. 1991).

Adult NT-3 protein levels range from 1 to 1.5 ng/g wet weight with hippocampus, cerebellum, and piriform cortex having the highest levels and superficial and deep cortical layers containing the greatest immunoreactivity (Anderson et al. 1994). Acid treatment has been shown to increase NT-3 detection by 30–50 %, depending on the region (Okragly and Haak-Frendscho 1997). NT-3 is detected in the internal granule cell layer of the cerebellum and ELISA estimates the concentration is around 500 pg/g wet weight in mature rats (Söderström and Ebendal 1995). Levels in neonatal animals reach 9 ng/g wet weight transiently in cingulate cortex (2.5 ng/g in hippocampus) and peak expression in the hippocampus, cerebellum, piriform cortex, and entorhinal cortex occurs at birth and declines over the first 2 postnatal weeks (Ernfors et al. 1990b; Söderström and Ebendal 1995; Das et al. 2001).

NT-3 regulates the development of discrete neuronal populations during early, middle, and late periods of CNS neurogenesis and synaptogenesis. NT-3 regulates neural crest development and is expressed at the earliest steps in neurulation (Maisonpierre et al. 1990b). NT-3 has a developmental peak in mRNA expression that coincides with the arrival of perforant path afferents into the hippocampus (Maisonpierre et al. 1990b, Ernfors 1990b; Friedman et al. 1991; Lauterborn et al. 1994), and NT-3 is transiently expressed in the hilus of the hippocampus at birth (Friedman et al. 1999). Similarly, late expression of NT-3 mRNA in the visual cortex and lateral geniculate nucleus coincides with eye opening and decreases after the critical period for ocular dominance column formation to undetectable levels in the adult (Lein et al. 2000). Follow-up studies using Cre-mediated deletion in cortex establish a role for NT-3 in thalamocortical innervation (Ma et al. 2002). NT-3 expression in the thalamus is transient, and targeted deletion of NT-3 in the mouse cortex selectively perturbs the development of thalamocortical axons projecting to retrosplenial and visual cortex (Ma et al. 2002). In organotypic cultures, NT-3 induces cortical, axon fiber branching and is an attractive signal for layer 6 axons while repelling layers 2/3 axons (Castellani and Bolz 1999).

Cerebellar granule neurons switch trophism from BDNF to NT-3 after they enter the internal granule layer (Segal et al. 1992). Conditional deletion of NT-3 results in a dramatic reduction in foliation of the cerebellum with normal Purkinje cell arborization, normal EGL proliferation, and normal lamination (Bates et al. 1999). Deletion of IGF-1 receptor in the cerebellum reduces NT-3 and results in a similar foliation phenotype (de la Monte et al. 2011). In addition to scattered pre- and post-migratory cerebellar granule cells expressing mRNA (Rocamora et al. 1996; Ernfors et al. 1990a), NT-3 immunoreactivity is detected in cerebellar Purkinje cells, cerebellar interneurons, deep cerebellar nuclei, medial vestibular nuclei, and the inferior olive (Friedman et al. 1991; Zhou and Rush 1994). Purkinje cells have not been shown to express NT-3 mRNA (Ceccatelli et al. 1991; Ernfors et al. 1994a), suggesting that NT-3 may accumulate in Purkinje cells via retrograde or anterograde transport.

TrkC has limited distribution in the CNS of mature animals but is highly expressed early in development. TrkC binding, TrkC mRNA, and NT-3 expression are similar, with the highest receptor levels in superficial cortical layers, stratum oriens, stratum radiatum, the molecular layer of the dentate gyrus, the nucleus of the

lateral olfactory tract, entorhinal cortex, the anterior olfactory nucleus, and the anteromedial thalamic nucleus. Neocortical deep layers, striatum, amygdala, dorsal root ganglia, and the central gray of spinal cord show moderate iodinated-NT-3 binding and TrkC expression, while expression is low in the cerebellum (Altar et al. 1993, 1994; Tessarollo et al. 1993).

2.3.2 NT-3 and Ethanol: Animal Models

It is not surprising that few studies have examined the effect of ethanol on NT-3/TrkC expression after ethanol exposure in mature animals, given the low, limited, and discrete regional expression in the adult. Recent studies of human postmortem tissue have suggested a role for ectopic induction of NT-3 induced by oxidative stress in the etiology of autism, particularly in cerebellar dysfunction (Sajdel-Sulkowska et al. 2011), but studies on NT-3 in developing and adult animals have been equivocal. NT-3 administered into the ventricles did not rescue vasoactive intestinal peptide-immunoreactive neurons in the suprachiasmic nucleus of withdrawn animals (Paula-Barbosa et al. 2003). Similarly, Baek and colleagues (1996) did no observe any changes in hippocampal NT-3 mRNA normalized to cyclophilin A after 28 weeks of constant ethanol exposure via liquid diet. Miller (2004) used a repeated episodic vapor exposure model, while Miller and Mooney (2004) exposed rats chronically to ethanol via liquid diet for 8 or 24 weeks and measured NT-3 levels by ELISA in parietal and entorhinal cortices, hippocampus, septum, and basal forebrain. Episodic exposure increased hippocampal NT-3 levels (from ~100 to 155–195 pg/mg protein) and reduced in basal forebrain NT-3 (from ~500 pg/mg protein to ~200 pg/mg). Chronic exposure increased NT-3 in entorhinal cortex and septum at both 8 and 24 weeks but transiently increased levels in basal nuclei and hippocampus. Both Miller and Mooney (2004) and Miller (2004) report the highest NT-3 content in the septum (600–700 pg/mg protein) with hippocampal levels ranging from 100 to 150 pg/mg protein and entorhinal cortex slightly higher at ~200 pg/mg protein. These data are given normalized to total protein but assuming 10 % protein, a value of 100 pg/mg is roughly 10 ng/g wet weight and regions such as septum contain 45+ ng/g wet weight. It should be noted that these concentrations are higher and not in the regional proportions previously observed in the adult, even upon acid treatment (see above) but may be the result of different sample preparation methods or, alternatively, significant transport.

When measured in homogenates after a variety of exposure paradigms, ethanol exposure either prenatally or postnatally changed NT-3 mRNA (Fattori et al. 2008; Light et al. 2001; Tsuji et al. 2008) and protein levels measured by ELISA (Heaton et al. 2000c). Heaton and colleagues (2000c) showed a trend toward increased "cortex/striatum" NT-3 on PN10 with PN4-10 vapor exposure (~90 pg/g wet weight to ~140 pg/g wet weight) in pups, but this did not reach statistical significance and NT-3 levels did not differ in other regions examined. When the telencephalon was further dissected (Heaton et al. 2003b), exposure to ethanol at PN14, but not the vulnerable PN3 exposure, increased NT-3 from ~8 ng/g to ~14 ng/g of striatal wet weight in rats at 24 h after exposure; NT-3 levels did not vary between

PN3 and PN14 (Heaton et al. 2003b). These levels are perplexing, given the variable concentrations observed between experiments and the lack of a developmental decrease in NT-3 postnatally (i.e., Das et al. 2001). NT-3 mRNA and NT-3 promoter–driven LacZ is not detected in the striatum in developing or mature animals (Maisonpierre et al. 1990b; Vigers et al. 2000; Soderstrom and Ebendal 1995; Das et al. 2001; Ernfors 1990b; Friedman et al. 1991; Lauterborn et al. 1994). Moreover, mRNA and LacZ expression also indicate low level of expression from the resident cells in the septum and basal forebrain. Therefore, the source of striatal NT-3 is unclear in these experiments as well. As discussed above, NT-3 is transiently produced in many structures including the thalamus, visual, cingulate, and retrosplenial cortex. NT-3 also regulates thalamocortical axon development during the critical stage with a peak in mRNA expression at PN8 (Ma et al. 2002). Again, NT-3 protein measured in striatum could be in retrograde transit through the striatum since the PN3 and PN14 time points in Heaton (2003b) flank the peak at PN5-8 but levels have not been shown to vary by orders of magnitude within a few days developmentally.

When the cerebellum is subdivided or immunoreactivity evaluated in situ, NT-3 and TrkC are sensitive to ethanol exposure in selected regions. TrkC immunoreactivity is reduced when examined specifically on Purkinje cells in pups exposed to ethanol during the vulnerable PN4-6 window (Light et al. 2002), but global mRNA for TrkC, likely representing mRNA from mature granule cells as well, was unchanged (Light et al. 2001). When the vermis was selectively assayed by ELISA, Parks and colleagues (2008) found increased cerebellar NT-3 and an increase in hippocampal NT-3 in mature animals after prenatal exposure, which could reflect compensation within the system. The high dose (6 g/kg/day by gavage) increased NT-3 in both regions while only the vermis was sensitive to the lower dose (4 g/kg/day). In addition, NT-3 levels in the vermis were higher in animals exposed to an enriched environment after prenatal alcohol exposure at both doses, again suggestive of a role in plasticity. The NT-3 concentrations measured were again higher than earlier reports in normal rats, but the relative levels, with the hippocampus having the highest NT-3 content, followed by cerebellum, were consistent.

The discrepancy between these studies and the classical descriptions is unclear as is the source of the high NT-3 levels measured in tissues not shown previously to have high levels of NT-3, suggesting the source of the NT-3 may be external to the structure. Acid treatment has been shown to increase NT-3 detection, but this should be uniform and not change the relative levels from region to region. Neutralization can also cause the reformation of NGF protein complexes. The samples in Miller and Mooney (2004) and Miller (2004) were fractionated by centrifugation, which may have concentrated specific pools of NT-3, and acid treated prior to assay; the samples in the Heaton studies were also acid treated. Parks and colleagues report higher levels than previously observed, but ratios consistent with the classical studies and those using in acid treated/neutralized samples in normal rodents.

2.3.3 NT-3 and Ethanol: In Vitro Models

The role of NT-3 in development is similarly time- and population-selective, and the effect of NT-3 on ethanol toxicity is similarly selective. Seabold and colleagues (1998) did not observe a protective effect of NT-3 on differentiated cortical neurons treated in vitro with a high (400 mg/dL) concentration of ethanol. Earlier in development, exposure of embryo cultures to ethanol for 3–10 h in vitro reduced proliferation of cephalic neural crest cells that was prevented by the addition of NT-3 to the medium (Jaurena et al. 2011). These studies are consistent with the limited cortical responsiveness to NT-3 and the early developmental peak in expression (see Sect. 2.3). Recent studies indicate that NT-3 may have a larger role in cerebellar development than previously appreciated. Cerebellar foliation is impaired when the insulin or IGF-1 system is perturbed in the cerebellum by siRNA; NT-3 levels are also reduced (de la Monte et al. 2011). This treatment reduces cerebellar foliation, suggesting role for NT-3 in this process, and developmental ethanol exposure similarly impairs cerebellar foliation (Cragg and Phillips 1985; Sakata-Haga et al. 2001).

3 Nonclassical Neurotrophic Factors: IGF-1, GDNF, and PACAP

3.1 The Insulin Family: IGF and ALK

IGF-1 is known to be involved in ethanol toxicity both centrally and peripherally. IGF-1 is produced in the liver in response to growth hormone secretion from the pituitary and thereby links IGF-1 levels to ethanol-induced growth restriction. Circulating IGF-1 levels in adults may also be involved in ethanol-induced vascular damage due to a lack of IGF-1-mediated repair, either directly by inhibiting signaling or secondary to hepatotoxicity, thereby compromising the blood–brain barrier. IGF-1 is bound in vivo to IGF Binding Proteins (IGFBPs) that further regulate bioavailability. Acute ethanol exposure results in a dramatic increase in IGFBP-1 and a decline in IGF-1 in healthy adults, while chronic ethanol exposure decreases IGF-1 bioavailability and growth hormone levels in patients (Passilta et al. 1999; Rojdmark et al. 2000; Rojdmark and Brismar 2001). After 4 days of withdrawal, IGF levels increase and IGFBP-1 levels decrease (Passilta et al. 1998). IGF-I and IGF-II are both upregulated in children exposed prenatally to ethanol, indicative of compensatory induction and a potential biomarker for FAS/ARND (Aros et al. 2011). The significance of alterations in peripheral IGF-1 to brain pathology is uncertain, but IGF-1 crosses the blood–brain barrier (Pan and Kastin 2000) and transport is activity dependent (Nishijima et al. 2010). Moreover, reduced IGF-1 levels are associated with depressive-like behavior (Mitschelen et al. 2011). Circulating IGF-1 levels are sensitive to hepatic function, thereby providing a link between hepatotoxicity and neuronal toxicity and repair processes. The GH-IGF-1 axis, neural activity, vascular integrity, and hepatic function are intimately linked, particularly during development.

IGF-1 is active in the early stages of neurogenesis, regulates stem cell proliferation, and enhances survival after ethanol exposure (Tateno et al. 2004). Ethanol blocks IGF-1-stimulated proliferation in vitro (Resnicoff et al. 1996) and ERK activation (Hallak et al. 2001) but, while trophic, does not significantly protect cerebellar granule cells from ethanol toxicity (Bonthius et al. 2003). Interestingly, in vivo siRNA-mediated reduction in insulin or IGF-1 receptor signaling produces cerebellar malformations that resemble fetal alcohol syndrome (de la Monte et al. 2011). McClure and colleagues (2011) used *Drosophila* to model fetal alcohol syndrome and implicate *Drosophila* insulin-like peptide and reduced insulin receptor expression in the teratogenic effects of ethanol. Another related molecule, ALK, was also recently suggested to be ethanol-sensitive in fly and mouse experiments (Lasek et al. 2011a, b). ALK is proapoptotic in the absence of ligand, but signals survival in presence of ligand (Yanagisawa et al. 2010). ALK promotes sensitization and cocaine reward (Lasek et al. 2011a) in flies and is involved in ethanol tolerance and reward in mice (Lasek et al. 2011a, b). ALK knockout mice show enhanced loss of righting reflex in response to high doses of ethanol, and this is not explained by reduced ethanol metabolism. ALK expression in striatum is negatively correlated with ethanol sensitivity. While knockout mice were more sensitive to the sedating effects, they were less sensitive to ataxia. These data implicate multiple intermediates in the IGF pathway as potential modulators of ethanol sensitivity and toxicity.

3.2 GDNF

GDNF is also a target derived factor that was originally described as a trophic factor for dopaminergic, sensory, and motor neurons (reviewed by Pascual 2011). GDNF is a member of a larger family that includes Artemin, Neurturin, and Persephin. While trophic for dopaminergic neurons, genetic ablation of GDNF does not affect genesis or survival of dopaminergic neurons (Sanchez et al. 1996; Jain et al. 2006), but Ret deletion cause late degeneration of the substantia nigra pars compacta (Kramer et al. 2007). GDNF is also produced in peripheral tissues, with high levels detected in kidney, ovary, testes, stomach, skin, and lung (Trupp et al. 1995). Like neurotrophins, GDNF signals through tyrosine kinase coupled pathway. GFR-alpha 1 binds GDNF and recruits the Ret tyrosine kinase; neurturin preferentially activates alpha 2, and artemin preferentially activates alpha 3 (reviewed by Mason 2000). Ret activation leads to ERK, Akt, phospholipase C, and Src activation to promote survival and differentiation. GDNF is also a potent regulator of anti-apoptosis pathways and NFkB. As with classical neurotrophins, alternative signaling pathways also exist for GDNF. GDNF can signal through NCAM to activate Fyn and focal adhesion kinase which mediate neurite outgrowth and survival (Sariola and Saarma 2003). Homophilic transsynaptic binding of GFR-alpha has also been described during hippocampal synaptogenesis (Ledda 2007).

GDNF is expressed at high levels in the striatal fast spiking interneurons, olfactory bulb, ventral mesencephalon, and septum with lower expression in the cerebellum and colliculi (see Fig. 1 and Hidalgo-Figueroa et al. 2012;

Pochon et al. 1997; Golden et al. 1999 for detailed anatomy). Receptors for GDNF are more widely expressed with Ret mRNA detected at high levels in diencephalic structures, cerebellum, brain stem, and olfactory bulb. Ret expression is low in the hippocampus, cortex, and striatum. GFR-alpha 1 is expressed at high levels in the septum, hippocampus, and colliculi, but at lower levels in the olfactory bulb, diencephalon, and brainstem. GFR-alpha 2 is high in cortex, hypothalamus, and colliculi, with low expression in the cerebellum and brainstem. GFR-alpha 3 expression is limited to the developing CNS and cerebellum (Masure et al. 1998) but is expressed in peripheral tissues such as heart, lung, and trigeminal ganglia. Interestingly, the striatal FSIs, not medium spiny neurons, produce GDNF while the receptors are produced by, and GDNF is accumulated in, mesencephalic dopamine neurons (Hidalgo-Figueroa et al. 2012; Barroso-Chinea et al. 2005). As with other neurotrophins, the differential distribution of GDNF and its receptors is consistent with target-derived factors and the consumer-producer model of neurotrophin action.

As with BDNF, GDNF was also identified for having the ability to reduce alcohol consumption in rodents (Carnicella et al. 2008, 2009a, b). The locus for this effect is the VTA since infusion of GDNF shRNA into the VTA blocks the effect (Barak et al. 2011) so it does not appear to involve fast spiking interneuron in the striatum, which also produce GDNF. Examination of the effects of ethanol on GDNF-mediated survival is limited. Fiore and colleagues (2009b) observed a decrease in cortical GDNF after a diet of chronic red wine (but not ethanol). Hippocampal GDNF does not appear sensitive to ethanol in mature animals (Okamoto et al. 2006).

Developmental studies in vivo indicate that GDNF protects developing chick spinal motor neurons from ethanol, but ethanol exposure did not reduce muscle GDNF levels in rats (Bradley et al. 1999; Barrow Heaton et al. 1999). However, Wentzel and Ericksson (2009) found a decrease in GDNF in trunk, but not cranial, neural crest–derived cells from the E10 rat embryo. Interestingly, this paper also reported a decrease in GAPDH mRNA in cranial neural crest cells exposed to ethanol. McAlhany and colleagues (1999) also found a reduction in GDNF protein, but not mRNA, with developmental ethanol exposure but a paradoxical increase in GDNF signaling to Shc. Neonatal brain growth restriction in response to high dose (5 g/kg gavage) ethanol during the postnatal brain growth spurt (PN6-8) is similarly correlated with a transient reduction in GDNF (Tsuji et al. 2008). (Exposure on PN 2–4 or PN 13–15 also reduced BDNF levels, but this did not correlate with microcephaly, as discussed above.) Interestingly, the ERK pathway was similarly sensitive regardless of age but inhibition of the Akt pathway was only observed with PN6-8 exposure, correlating with the temporal decrease in GDNF in these experiments.

In vitro, GDNF reverses Purkinje cell ethanol toxicity in cerebellar explants (McAlhany et al. 1997, 1999) and ethanol blocks GDNF-stimulated process outgrowth in granule cells (Chen and Charness 2012). GDNF reversed ethanol-induced JNK activation in SK-N-SH neuroblastoma cells and associated cell death (McAlhany et al. 2000). Interestingly, these cells underwent DNA fragmentation at 34 and 68 mM ethanol but displayed signs of necrosis at concentrations above 100 mM. GDNF can also protect glial-type transformed cells from ethanol toxicity

in vitro (Villegas et al. 2006). In contrast, Mitchell and colleagues (1999) found no neuroprotective effects of GDNF against cell death in hippocampal cultures, induced by high concentrations (>100 mM, 0.4–1.6 g/dl) of ethanol combined with hypoglycemia and hypoxia.

3.3 PACAP

PACAP is a member of the vasoactive intestinal peptide/glucagon/growth hormone releasing hormone superfamily and is active in stress, anxiety, consummatory behavior, metabolism, and thermoregulation (Adams et al. 2008). PACAP receptors exists as splice variants that stimulate adenylate cyclase and phosphoinositol hydrolysis with different kinetics (Spengler et al. 1993). PACAP also signals through Ca^{++}, PCK; Src family kinases, and by transactivation of Trks through the PAC1 receptor, with Gq-coupled pathways predominating in the hippocampus (Macdonald et al. 2005; Rajagopal et al. 2004; Shi et al. 2010). PACAP may also regulate BDNF levels through receptor-coupled activation of RACK1 through cAMP (Yaka et al. 2003a) placing BDNF downstream of PACAP. PACAP-deficient mice display hyperlocomotion, decreased body mass, novelty-seeking behavior, jumping, impaired hippocampal associative learning, and mossy fiber LTP. PACAP in the arcuate nucleus of the hypothalamus stimulates NPY neurons to regulate carbohydrate intake (Nakata et al. 2004) but inhibits food intake via the POMC arcuate neurons (Mounien et al. 2009). Peripherally, PACAP is coupled to catecholamine secretion from adrenal chromaffin, providing a peripheral link to the stress response (Kuri et al. 2009).

The initial evidence for involvement of PACAP in ethanol sensitivity came from *Drosophila* where an allele of the *Drosophila* PACAP homologue amnesiac called cheapdate was identified as a gene regulating ethanol sensitivity (Moore et al. 1998). Rutabaga, another cAMP pathway mutant, is required in mushroom bodies for ethanol consumption in *Drosophila* (Xu et al. 2012). As with flies, PACAP knockout mice are less sensitive to the hypothermic and hypnotic effects of ethanol (Tanaka et al. 2004). Cerebellar granule cells from PACAP knockout mice are more sensitive to ethanol and peroxide toxicity, while addition of PACAP or dibutryl cyclic AMP reversed this effect (Vaudry et al. 2005). PACAP administered to neonatal rats mitigated the ethanol-induced loss of righting and apoptosis, possibly through caspase 3 and cJun inhibition (Botia et al. 2011). As with BDNF, PACAP also protected cerebellar granule cells from ethanol toxicity by the activation of PI3K (Bhave and Hoffman 2004).

3.4 Nonconventional Neurotrophic Factors in Patients with Alcohol Dependence

To date only one study has examined peripheral GDNF levels in alcohol-dependent patients. Heberlein and colleagues (2010) found GDNF levels significantly

depressed in the serum of alcohol-dependent patients which remain decreased for up to 2 weeks after withdrawal. Interestingly, serum levels were also inversely related to subjective assessment of tolerance. Those with high serum GDNF levels reported lower tolerance and may therefore be more likely to limit their alcohol intake. This is consistent with observations in rodents, suggesting that GDNF negatively regulates ethanol consumption (Carnicella et al. 2009a, b).

4 Neurotrophic Therapies in Alcoholism, FAS, and ARND

Neurotrophins-based therapies for both addiction and repair of ethanol-induced brain damage may be on the horizon. Small interfering RNA and other expression-based strategies have been used experimentally to dissect the pathways involved in ethanol self-administration in rodents, but this strategy will not be immediately useful for treating patients. Direct activation of Trks with ICV neurotrophin infusion has been used experimentally. For example, neurotrophin infusion can maintain ethanol tolerance (Szabò and Hoffman 1995) and infusion of NGF can restore cholinergic function in the mature brain after chronic ethanol exposure in rodents (Cadete-Leite et al. 2003; Lukoyanov et al. 2003). Unfortunately, ICV infusion of NGF is also not feasible for human treatment, but intranasal delivery of IGF-1 is being investigated for ameliorating ischemia and hypoxia-induced damage in developing mice (Lin et al. 2009, 2011) and in adult stroke models (Kooijman et al. 2009). Intranasal IGF-1 also protects Purkinje cells in spinocerebellar ataxia type 1 and improves roto-rod function (Vig et al. 2006). Given the trophic role of IGF-1 in the CNS, IGF-1 might also be useful in FAS/ARND and it readily crosses the blood–brain barrier. While not protective against the insult, IGF-1 may enhance recovery in the child.

Neurotrophic peptides and peptidomimetics are also being developed, which may reverse ethanol-induced neurodegeneration and withdrawal-associated anxiety. Cyclotraxin-B was recently isolated from a peptidomimetic screen as a TrkB allosteric antagonist that reduces anxiety-like behavior (Cazorla et al. 2010, 2011). Such molecules may reduce anxiety-associated drinking and withdrawal hyperexcitability but may also pose a toxicity risk. Conversely, mimetic and small molecule agonists of TrkA and TrkB reverse cell death and enhance recovery in experimental models (Scarpi et al. 2012; Massa et al. 2010; Jang et al. 2010) and may therefore be useful for treating ethanol-induced neurodegeneration and cognitive dysfunction. Activity-Dependent Neurotrophic Factor and related peptides enhance survival in moderate (Zhou et al. 2008) and extreme (Incerti et al. 2010) fetal alcohol models. Interestingly, the ADNF peptides SAL and NAP reversed the deficits observed after a single exposure on day 8 of gestation to a dose of ethanol that produces BACs in excess of 500 mg/dL. This protective effect was attributed to normalization of ethanol-induced upregulation of BDNF at E9 and E18. Thus, NAP and SAL can influence events prior to the onset of CNS neurogenesis and normalize BDNF expression without off target teratogenesis; however, recent studies have excluded a direct role for BDNF in NAP effects on axon formation

(Chen and Charness 2012). The efficacy of NAP and related peptides may therefore be related to cytoskeletal interactions (Gozes 2011), or inhibition of apoptosis (Zemlyak et al. 2009) and not a direct interaction with BDNF signaling.

Small molecule pharmacological strategies that indirectly modulate neurotrophin levels have also been proposed and tested in rodent models. Antidepressants increase BDNF in the hippocampus and can reverse ethanol-induced reductions in BDNF in the hippocampus (Hauser et al. 2011), which may increase recovery after long-term ethanol exposure. The anti-addiction compounds Ibogaine and noribogaine may also interact with GDNF to reduce ethanol drinking (He et al. 2005; Carnicella et al. 2010). Neurotrophin-based therapeutics need not involve administration of a drug, however. Exercise in mature animals can induce hippocampal neurogenesis and increase BDNF levels, which ameliorates many of the effects of prenatal ethanol exposure (Boehme et al. 2011). Antioxidants provide neuroprotection in fetal alcohol models, in part through modulation of neurotrophins (reviewed by Brocardo et al. 2011). Dietary supplementation with omega-3 fatty acids also reverses behavioral deficits in fetal alcohol models and increases BDNF, possibly through antioxidant modulation (Patten et al. 2012). Similarly, choline supplementation modulates neurotrophins and choline is being examined in preclinical models as a treatment for alcohol-related birth defects (Ryan et al. 2008; Monk et al. 2012). These interventions, while subtle, may increase neurogenesis and plasticity, ultimately involving regulation of neurotrophins. The strategies vary but all ultimately increase neurotrophic activity to influence survival and plasticity.

5 Conclusion

Alterations in neurotrophins and neurotrophin signaling mediate the acute toxic effects, plasticity that occurs with pharmacodynamic tolerance, plasticity that occurs with addiction, and repair that occurs after withdrawal. The temporal and cell specific activation and inhibition of neurotrophins and their associated pathways have not been conclusively determined, and the data on ethanol and neurotrophic factors summarized in this chapter clearly do not outline a cohesive story. Neurotrophin levels can increase, decrease, or not change depending on the brain region, time of exposure, and presence or absence of drug upon analysis. Neurotrophin signaling is dependent upon concentration, with full-length receptors desensitizing, high concentrations activating p75, and related receptors being nonspecifically activated at higher concentrations. In addition, neurotrophin levels and signaling to not occur in isolation; therefore, it is important to consider the physiological changes that result from neurotrophin action. For example, an increase in glutamatergic synaptic activity during withdrawal would be expected to increase BDNF but the place of BDNF in this sequence is not well defined in most studies. Does BDNF drive these changes or, more likely, does BDNF increase as a result of excess NMDA receptor activation? The sequence is different early in exposure and with low ethanol concentrations where ethanol can reduce signaling

in BDNF independent of effects on ion channels (Kolb et al. 2005; Ohrtman et al. 2006). In this case, neurotrophin signaling may be directly involved in the regulation of subsequent events and not vice versa. When concentrations of ethanol become too high, none of these adaptive responses are likely to occur, however, as is observed with suprapharmacological concentrations. More subtle effects are likely to contribute to ethanol-induced teratogenesis and chronic toxicity.

The expression data for receptors and neurotrophin levels measured after ethanol exposure also frequently do not agree. It is possible that ethanol induces ectopic expression in specific cell types, but this has not been specifically addressed since the control cells in these regions also frequently show expression of neurotrophins they are not believed to express. This may be partially due to the fixation procedures used to detect immunoreactivity in situ since neurotrophins are difficult to detect with immunohistochemistry. In situ hybridization and protein detection with and without colchicine treatment and global mapping may also help resolve some of these discrepancies and locate the "producer" neurons where neurotrophins are sensitive to ethanol treatment. The availability of genetically tagged mice will also aid in dissecting these issues anatomically, functionally, and allow for separation of specific populations by cell sorting. In addition, Cre-recombinase-mediated deletion in specific cell types, for example, BDNF deletion in cortex versus medium spiny neurons, could be combined with self-administration behavioral experiments to define the loci for specific effects. The pharmacological tools described in the previous section will aid in dissection of the actions of neurotrophins in ethanol toxicity, tolerance, addiction, withdrawal, and the development of FAS/ARND.

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