Chapter 5
Mechanisms of Stress-Dependent Neuroinflammation and Their Implications for Understanding Consequences of Alcohol Exposure

Terrence Deak, Anny Okrainets, and Tamara L. Doremus-Fitzwater

5.1 Introduction

Central inflammatory processes evoked by immunological challenges are responsible for directing a whole host of neural, hormonal and behavioral reactions in order to defend against infection and initiate recuperative processes. In particular, activation of brain cytokines appear especially important to the induction of sickness behaviors that are meant to help coordinate recovery during infection. More recently, research has shown that exposure to stressors may also activate peripheral and brain immune processes, with activation/inhibition of central cytokines being a key component of this response. Specifically, dynamic changes in central cytokines and other key inflammatory signaling pathways have been demonstrated to ultimately result in the expression of sickness behaviors as well, suggesting that illness- and stress-related neural consequences may share common mechanisms. Limited studies have now begun to show that alcohol is yet another exogenous stimulus that may also influence central cytokines. In addition to modulating behavioral responses incurred by alcohol exposure, early changes in cytokines may portend the development of long-term neuropathological consequences associated with chronic alcohol exposure. The goal of this chapter, therefore, is to review the literature surrounding stress-related neuroinflammation and alcohol-related changes in cytokines as a means for understanding the complex interaction between stress-responsive systems, neuroinflammatory processes, and their interactions with alcohol exposure.

Subsequently, the influence of alcohol exposure on the expression of cytokines will be addressed. Acute or chronic alcohol administration and its consequent withdrawal have been shown to alter cytokines, as well as to modulate the immune response to an

T. Deak, Ph.D. (✉) • A. Okrainets • T.L. Doremus-Fitzwater
Behavioral Neuroscience Program, Department of Psychology, Binghamton University, State University of New York, PO Box 6000, Binghamton, NY 13902-6000, USA
e-mail: tdeak@binghamton.edu

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immunological challenge. Though limited thus far, what literature is available would suggest that alcohol exposure has the capacity to significantly change both peripheral and central cytokines that could, in turn, ultimately impact behavior, stress-related processes, and (under long-term exposure circumstances) alcohol-associated brain/tissue damage. Alcohol-induced cytokine alterations may also interact with other processes more traditionally associated with stress challenges, such as the sympathetic nervous system (SNS) and hypothalamic–pituitary–adrenal (HPA) axis, which could either positively or negatively, respectively, feed back onto cytokine signaling.

Before proceeding, however, it is necessary to acknowledge from the outset a few critical distinctions. For instance, the field of stress research readily accepts the view that immunological challenges produced by antigen, pathogen, or direct injection of cytokines represent one distinct form of a "stress challenge" (e.g., [10]). This issue is well established by empirical studies demonstrating that immune activation leads to activation of the HPA axis (e.g., see [11, 12]) and SNS activity (e.g., see [13]) in much the same way as exposure to psychological and/or physical stress challenges that are otherwise devoid of antigenic or pathogenic components. However, because the impact of antigen or pathogen exposure on cytokine expression in brain is largely to be expected, use of the term "stress" in the present context will specifically exclude challenges that are, by their very nature, immunologically based. In this regard, much of what we describe as stress-related neuroinflammation really falls into the category of what the field is now referring to as "sterile inflammation," which simply refers to induction of inflammatory processes in the absence of antigen/pathogen/wound exposure.

The second semantic issue we would like to address from the outset is what is meant by the term "neuroinflammation" or "inflammatory processes in brain." This is a critical issue because, at some point, one would like to extrapolate from isolated changes in a specific cytokine (such as interleukin-1; IL-1), cellular process (e.g., morphological changes in microglia), or physiological condition (e.g., hyperthermia) toward these broader constructs. Though we will seek to avoid use of terms like this that tend to amalgamate specific features of the inflammatory response into broader statements, it will be occasionally necessary to make such extrapolations in order to synthesize the diverse range of inflammatory changes examined in brain after stress into some meaningful portrait of the relationship between stress, neuroinflammation, and larger issues surrounding central nervous system (CNS) dysfunction/function.

5.2 Stress and Neuroinflammation

5.2.1 Cytokines in the CNS Coordinate the Expression of Sickness Behaviors

Cytokines are a classification of small proteins, which are important for regulation of cell signaling processes within the immune system. Comprised of chemokines, interleukins, and lymphokines, these molecules are critical for initiation of the

immune response to infection or damage and are secreted by several cell types within the CNS, including microglia, perivascular and meningeal macrophages [14], astrocytes [15], and even neurons [16]. Additionally, cytokine receptors have been identified on virtually all cell types within the CNS, and there is evidence to suggest that genes coding for cytokines are expressed in both neurons and glia in the brain under nonpathological conditions [17].

Following infection, it has been consistently shown that one especially important consequence of immune activation is expression of sickness behaviors. Studies have demonstrated that acute illness induced by LPS administration or other immunogens produces a constellation of classic behavioral alterations including reduced social and sexual interaction, decreased exploration in a novel environment, reduced overall activity, hyperalgesia, and suppressed food and water intake [18–25]. Collectively, these behavioral responses to acute illness are referred to as "sickness behaviors" [21] and, in combination with the physiological responses to infection (e.g., fever), are thought to act in an adaptive manner to promote efficient recovery from infection [21, 26]. Increased central expression of immune-derived factors, including the proinflammatory cytokines IL-1, tumor necrosis factor (TNF-α), and interleukin-6 (IL-6), appears to coordinate the manifestation of sickness behaviors, with IL-1 playing a particularly prominent role in their initiation. For instance, many sickness behaviors can be produced by central administration of IL-1 [24, 27, 28], whereas inhibition of central IL-1 activity blocks many of the sickness responses normally observed following peripheral immune activation [25]. At a mechanistic level, cytokine signals originating in peripheral organs such as the liver, gut, and spleen are communicated to the CNS through a variety of pathways (for a review, see [29]), ultimately leading to increased expression of proinflammatory cytokines in the CNS. Interestingly, both neurons and glia express cytokines under varying conditions [16, 30], and the hypothalamus appears to be one crucial CNS structure where proinflammatory cytokines precipitate the behavioral sickness response [21, 31–33].

In contrast, research has shown that anti-inflammatory cytokines in the CNS inhibit sickness behaviors and suppress peripheral immunity. As with proinflammatory cytokines, there are of course numerous cytokines in the CNS that serve an anti-inflammatory role such as (but not limited to) interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1ra), interleukin-4 (IL-4), and interleukin-13 (IL-13) (for review, see [34]). Indeed, the central regulation of inflammatory processes—including sickness behaviors—is an intricate process involving a delicate balance between the expressions of both pro- and anti-inflammatory constituents in the CNS [35]. The anti-inflammatory cytokine, IL-10, has received a great deal of attention for its role in the CNS in counter-regulating the immune response (see [36] for a review). For instance, central administration of IL-10 blocks the reduction in social interaction produced by peripheral LPS administration [20] and inhibits the aphagia produced by central administration of the HIV envelope glycoprotein gp-120 [37]. IL-10 is also upregulated site-specifically within the CNS following immune activation, which is thought to prevent the development of certain neurodegenerative diseases with an inflammatory component [38]. The anti-inflammatory action of IL-10 is so powerful that researchers have recently employed adenoviral

delivery of IL-10 as a method for inhibiting neuropathic pain [39–41]. Thus, endogenous expression of IL-10 appears to play a unique role in counter-regulating centrally mediated neuroinflammation, and central injection of IL-10 inhibits the expression of sickness behaviors during acute illness.

Though IL-10 is one prominent example, there are a wide variety of endogenous peptides that have anti-inflammatory properties. For instance, alpha-melanocyte-stimulating hormone (alpha-MSH) has been shown to blunt or reverse inflammatory-related changes evoked by stress [42] and in response to other inflammatory conditions (for review, see [43]). Similarly, the IL-1 superfamily includes two members with properties that can be considered anti-inflammatory. The first member, IL-1ra, is an endogenous peptide ligand that binds to the IL-1 type I receptor without any known signaling consequences, thereby blocking the proinflammatory actions of endogenous IL-1. The second, IL-1 receptor type II, is a soluble receptor that, when released from the cell, binds to IL-1 and consequently prevents signaling of IL-1. Similar mechanisms of counter-regulation exist within the TNF family via TNF-binding protein—a soluble peptide that binds TNF-α and prevents interaction with its functional receptors. These hardwired mechanisms of regulation and counter-regulation among cytokine signaling systems emphasize the importance of understanding the complete inflammatory milieu under the conditions of interest, which has led to greater utilization of measurement techniques that permit analysis of multiple inflammatory factors within the same samples (e.g., [44]).

5.2.2 Historical Overview of Stress-Dependent Changes in Central Cytokines

Since cytokines are expressed at exquisitely low levels in the uninjured brain, isolation and detection of cytokines under nonpathological conditions has been difficult, therefore rendering the discovery of stress-induced alterations in cytokine levels problematic. Nevertheless, there is ample evidence that cytokines such as IL-1 serve a normal physiological function in brain. For instance, the expression of IL-1 in the CNS follows a circadian rhythm that is independent of corticosterone (CORT) secretion and appears to play a key role in the initiation of sleep [45–49]. IL-1 is also increased in brain after consumption of novel food in rodent models [50]. Furthermore, IL-1 signaling is self-propagating and requires very low levels of expression to exert functional consequences on target cells [17]. Thus, small fluctuations in cytokine expression tend to exert a disproportionately large impact on target cells, at least in part through feed-forward actions of cytokine-receptor interactions and the ability of local injections of cytokine within the CNS to affect cytokine expression at distal targets (e.g., [51–53]).

The impact of stress on cytokine expression and other inflammatory processes in brain has been an important line of inquiry due to its implications for disease states of the CNS and beyond. When it comes to stress-dependent changes in inflammatory processes, much more is known about IL-1 in brain and its modulation by stress than any other cytokine. For this reason, we will use these studies as a case example

of how stress exposure, and the hormonal systems activated by stress, interacts with inflammatory processes in brain. Although some initial papers in the mid-1990s reported that immobilization led to increased IL-1 expression using cellular techniques (in situ hybridization and immunohistochemistry), the bulk of what we know about IL-1 regulation by stress comes from studies utilizing gross brain dissections followed by IL-1 protein detection via ELISA. Using this strategy, for example, in one of our original studies, we demonstrated that adrenalectomized (ADX) subjects exhibited increased IL-1 protein in rat brain following inescapable tailshock relative to non-stressed ADX controls, with these stress effects not evident in sham-operated and stressed subjects [54]. At the time, these findings were important because they were the first to demonstrate that stressors were capable of significantly increasing IL-1 protein in brain. Within a few years, however, tissue extraction and assay sensitivity had improved to the point where it was no longer necessary to ADX rats in order to measure and detect tailshock-induced increases in IL-1 protein [45]. Furthermore, Nguyen et al. [45] also demonstrated the profound impact of endogenous CORT to inhibit brain cytokine responses evoked by stress, showing that both the magnitude and spatial distribution of IL-1 changes provoked by stress were augmented in ADX rats. Since then, our lab and others have gone on to successfully examine cytokine expression using gross dissections and ELISA detection (e.g., [22, 55–57]) or at the level of gene expression using RT-PCR [58–61], which together further support the view that stress challenges have the ability to drive cytokine changes in the uninjured brain. These changes, however, do not appear to be a universal response to all stress challenges [55, 61].

Indeed, through an exhaustive series of studies, we have examined IL-1 protein expression in the hypothalamus (a key stress-responsive site) following most commonly used laboratory stress challenges. This exploration was necessary in order to resolve discrepancies in the literature and address the highly appropriate critiques provided by experts in the field. Specifically, the primary issues we have worked to clarify can be summarized by three fundamental questions: first, does the increase in IL-1 produced by stress represent a universal response to all stress challenges? If this were the case, then we would expect systematic fluctuations in IL-1 (or other cytokines) in response to all stress challenges. Though early studies examining IL-1 protein failed to provide support for this viewpoint, our recent studies [61] may suggest otherwise. Secondly, is there a specific feature of the stress challenge (psychological distress, physical exertion, nociception) that is predictive of cytokine changes in brain? If this were true, then we would expect cytokine changes to vary across specific dimensions of the stress challenge in easily discernible ways. Such studies are ongoing in our laboratory. Finally, is there a threshold of stress that is necessary to increase cytokine expression in brain? This scenario is perhaps the most difficult to identify because, to our knowledge, there is no objective method for quantifying the intensity of a stress challenge, which would be essential for identification of a specific threshold.

Though we have no definitive answer to the conceptual issues raised above, we find them to be important theoretical considerations for understanding the relationship between stress and inflammatory processes in brain. Nevertheless, we have conducted a comprehensive series of experiments to try and tease these issues apart, as summarized below.

Based on studies focusing on IL-1 protein in whole hypothalamic blocks as the key measure of stress-related neuroinflammation, we know that stress challenges which would likely be characterized as psychological in nature such as social defeat [61], predator odor exposure [62], simple restraint in a Plexiglas tube [63], or maternal separation of guinea pig pups [64] have failed to alter hypothalamic IL-1 protein. Similar null effects have been observed following physiological stressors such as glucoprivic challenges evoked by injection of 2-deoxyglucose, or insulin-induced hypoglycemia [63]. However, when simple restraint in a Plexiglas tube was imposed on an orbital shaker as a method of "amplifying" the stress response (similar to [1, 63]), or combined with an insulin-induced hypoglycemia challenge [63], IL-1 was significantly increased in response to both "compound" stress challenges (restraint + oscillation; restraint + hypoglycemia), yet unaffected by the individual ones (restraint alone; hypoglycemia alone). These findings do not discriminate between a categorical model (point 2 above) and a threshold model (point 3 above), but they do provide evidence that substantive changes in IL-1 protein probably relate in meaningful ways to the severity of the stress challenge. Importantly, plasma CORT was examined in all of these studies and was not positively or negatively predictive of IL-1 changes [63]. This dissociation between IL-1 changes and CORT levels was true from simple observation of patterns in the data, as well as with more formalized statistical analyses performed later [65]. Additionally, one net outcome of these studies is that stress challenges involving exposure to an actively aversive stimulus (e.g., tailshock, footshock) appear to reliably produce telltale signs of inflammation such as elevated IL-1 [54, 61], which is in large part why many ongoing studies employ these types of stressors to pursue mechanisms of stress-dependent neuroinflammation (see [61] for further discussion).

As previously mentioned, the bulk of studies having examined IL-1 protein or mRNA changes following stressor exposure generally used gross tissue dissections of the hypothalamus, hippocampus, or cortex (e.g., [55, 56]), each of which are vastly heterogeneous structures. These early studies also indicated that IL-1 was probably the most stress-responsive of the classic proinflammatory cytokines in the CNS [56], an effect that we have validated more recently [61]. From these studies, IL-6 and TNF were either unaltered by stress exposure or were reduced as a result of stress, probably reflecting a CORT-mediated suppression (particularly for TNF-α). Given the need for greater spatial resolution (which would require enhanced sensitivity for measurement of cytokines in smaller tissue punches), we switched to RT-PCR for detection of inflammatory-related factors at the level of gene expression. This strategy additionally provided us with the advantage of being capable of examining numerous inflammatory factors within the same CNS structure. Accordingly, in a recent study [58], we examined a range of cytokines and other inflammatory markers in several brain structures after footshock, with a group of LPS-injected rats included as a control for assay sensitivity. Using this technique, it was again demonstrated that stress exposure resulted in significant IL-1 activation in the hypothalamus, as evidenced by increased IL-1 mRNA expression. Additional key outcomes of this experiment were that IL-1 changes in the hypothalamus were associated with increased CD14 expression (a cognate receptor for LPS), as well as decreased expression of CD200 receptor (which controls microglial activation state

via interactions with CD200 on neurons). Combined, these receptor-related changes suggest that microglia may be primed/activated by stress, which could ultimately account for priming effects that we and others had observed previously [6, 7, 9]. Moreover, these changes were restricted to the hypothalamus (no effects in hippocampus, cortex, or pituitary gland) and were highly reproducible (replicated in four successive experiments [58]).

Taken together, these gene expression changes intimated that increased IL-1 provoked by stress may be part of a larger stress-induced inflammatory process involving microglial activation. In support of this hypothesis, administration of minocycline (a tetracycline antibiotic that appears to selectively target microglia as an inhibitor) was found to block the increase in IL-1 protein [57] as well as other gene expression changes observed after footshock [58]. These findings complement prior studies suggesting that repeated restraint in mice led to microglial proliferation, which is often a downstream consequence of early microglial activation events [66]. Indeed, work from our lab and others has shown that stress exposure produces signs of microglial activation [58, 67], priming [6], and proliferation [66]. While these studies imply that microglia may be the cellular source of IL-1 produced by stress, there is also some evidence to suggest otherwise. For example, Kwon et al. [67] showed that repeated exposure to immobilization led to morphological alterations in microglia indicative of activation, yet their studies seemed to support the notion that IL-1 was expressed in neurons (not microglia or astrocytes) based on single-label immunohistochemistry. Another recent study also reported a similar localization of IL-6 in neurons after acute restraint in rats [68]. Thus, it seems likely that multiple cell types produce cytokines in response to stress, but the precise location, cell type, and cytokine being expressed may vary as a function of species, strain, prior experience, or the nature of the stress challenge imposed.

Regardless of whether IL-1 changes are expressed by neurons, astrocytes, or microglia, it is clear that IL-1 changes in response to stress (1) are some of the most widely observed and reproducible changes across stress challenges; (2) are associated with other, more subtle cellular changes indicative of neuroinflammation; and (3) will have a functional impact upon all cells in the local cellular microenvironment since IL-1 receptors are expressed broadly across cell type [69]. Whether the cellular source of cytokine changes produced by stress varies as a function of the nature or duration of the stress challenge or the cytokine being examined remains to be determined. Furthermore, despite these strong advances in our understanding of stress-related neuroinflammation, sensitivity of measurement and approach remain key challenges for the field. In particular, due to the low intrinsic expression of cytokines under these conditions, future studies utilizing immunohistochemical approaches and microdialysis to detect picogram quantities that are expressed/released at the cellular level will be critical for clarifying the functional relationship between stress and/or alcohol exposure and the functional role of cytokines in the CNS. Such determinations will have to be made with the strictest of controls and the highest sensitivity possible, with full acknowledgement that species, strain, stress challenge, and timing are likely to be key variables that significantly impact the outcomes and conclusions that are drawn.

Given the widespread effects that increased central inflammation may have, it is therefore important to understand how exactly stress exposure relates to inflammatory processes in brain and the consequences of stress-related neuroinflammation for CNS dysfunction/function. With this in mind, we will turn our attention toward mechanisms involved in regulation and counter-regulation of brain inflammatory responses evoked by stress. Ultimately, a clear understanding of the acute mechanisms by which stress leads to alterations in brain inflammatory processes will hopefully reveal novel insights and therapeutic targets for preventing the progressive impact of stress-dependent inflammation on CNS function and, relevant to the present context, provide guidance for understanding the involvement of neuroinflammation in alcohol-related behavioral adaptations and brain damage.

5.2.3 The Interaction Between Stress Hormones and Stress-Dependent Inflammatory Processes

Based on a long history of studies examining regulation of cytokines and other immunological processes by norepinephrine (NE) and CORT (reviewed more extensively below), and the obvious impact of stress challenges on these transmitter/hormonal systems, early studies examined the role of adrenergic receptor activation as a key driver of IL-1 changes produced by stress. For instance, intracerebroventricular (icv) injection of the β-adrenergic agonist isoproterenol [70] or other agents that facilitate noradrenergic transmission [71] markedly increase the expression of mRNA for IL-1 in the hypothalamus, an effect which may occur via receptors on microglia [72]. In this latter study, icv injections of isoproterenol increased mRNA for IL-1 in a number of hypothalamic nuclei, including the paraventricular, ventromedial, dorsomedial, and medial mammillary nuclei. A series of pharmacological studies performed by [60] showed that increased IL-1 protein in brain (hypothalamus, hippocampus, etc.) occurs via activation of β-adrenergic receptor activation since isoproterenol reproduced and propranolol reversed the effects of stress on IL-1 protein. These effects were synonymous with findings from our lab [57], which replicated and extended the pharmacology by showing that desipramine (a NE reuptake inhibitor) increased both basal and stress-evoked IL-1 concentrations in brain. Furthermore, while administration of α1-adrenergic receptor antagonists had no effect on stress-induced IL-1 expression in the CNS, these compounds completely reversed the increase in *plasma* cytokines produced by tailshock (Johnson et al. [60]). These intriguing findings suggest that plasma and brain cytokine responses may be controlled by different adrenergic receptor mechanisms, with α1 receptor activation modulating plasma cytokine changes and β-adrenergic receptor activation influencing central cytokine changes. Perhaps even more importantly, these findings showed that plasma cytokine changes are dissociable from the brain cytokine changes and support the notion that neither effect appears to depend on the other. This is a crucial separation given the bidirectional interactions that occur between central and peripheral immune processes [29]. However, it should be noted that elevations in plasma concentrations of cytokines are highly variable across

experiments and laboratories, making it difficult to establish clear functional relationships between plasma and brain cytokines. Whether this is due to low ambient expression, differential sample handling/processing, or the inherently unstable form of proteinaceous cytokines remains unclear.

Moving beyond pharmacological studies, the central mechanistic issues then turned toward neural circuits responsible for driving stress-dependent changes in IL-1 protein. With the NE system being the most logical target, Johnson et al. [60] injected the neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) into rats 3 weeks prior to tailshock exposure in order to selectively ablate central noradrenergic systems. This treatment was previously shown to target largely ascending adrenergic fibers through destruction of cells primarily in the locus coeruleus, which is a principle source of noradrenergic input to the hippocampus. Having used this procedure, NE levels in the hippocampus were reduced by approximately 87%, while the DSP-4 lesions only reduced NE levels in the hypothalamus by about 20%. The authors found that the lesion, as expected, blocked the increase in IL-1 protein in the hippocampus produced by tailshock, while stress-induced increases in IL-1 in the hypothalamus (where DSP-4 lesions had a lesser impact on NE levels) were largely unaffected by the lesion. It is probably important to note here that one key difference between studies conducted by Johnson's group and our lab is that their studies use Fischer rats, while our studies use Sprague Dawley rats. The net result of these subject differences is that Fisher rats show a much larger and broader spatial distribution of IL-1 changes in brain than Sprague Dawley rats. These strain differences are probably due to the well-documented, hyperadrenergic nature of Fisher rats relative to Sprague Dawley rats, as the hippocampus has not been a cytokine-responsive site in our studies (e.g., [55, 73]).

In order to more selectively target noradrenergic cells projecting largely from the nucleus tractus solitarius (NTS) to the hypothalamus and other forebrain structures, we recently performed a series of studies examining the impact of 6-hydroxydopamine lesions of the ventral noradrenergic bundle (VNAB) 10 days prior to stress exposure. As predicted based upon connectivity studies [74–77], this lesion led to approximately 70% depletion of NE in the paraventricular nucleus (PVN) and other structures, while other catecholamines were largely unaffected. Despite this NE depletion, rats bearing lesions of the VNAB unexpectedly exhibited an equivalent increase in IL-1 gene expression in the PVN after footshock relative to sham-lesioned controls (Blandino and Deak, unpublished observations). While these findings demonstrated that noradrenergic cells in the NTS likely do not modulate stress-induced increases in IL-1 in the hypothalamus, they do not conclusively determine the role of noradrenergic cells in the NTS as drivers of IL-1 changes in the PVN and suggest that the relationship between stress exposure and cytokine changes may be more complex than originally thought. Regardless, the evidence to date suggests that NE release is a key driver of inflammatory processes in at least some brain structures during times of stress.

In contrast to the actions of NE, which appear to stimulate the expression of IL-1 and other cytokines, CORT appears to constrain cytokine gene and protein expression during times of stress, at least in broad strokes. Indeed, there is a plethora of studies demonstrating that removal of endogenous glucocorticoids through ADX

[45, 54] or by injection of glucocorticoid synthesis inhibitors [58] enhances the expression of IL-1 and other cytokines in brain as a result of stress exposure. These findings parallel earlier studies showing that ADX enhanced the plasma IL-6 response evoked by exposure to a novel environment [78] and fit with the canonical viewpoint that glucocorticoids have powerful anti-inflammatory properties. While we do not wish to dispute this viewpoint, it is interesting to note that anti-inflammatory actions of CORT are most pronounced at high and supraphysiological concentrations, whereas lower concentrations of CORT appear to have some immune-potentiating effects (e.g., [6]). Whether these low-dose facilitation effects relate more directly to the timing of CORT injection relative to cytokine measurements, or represent differential tissue sensitivity to glucocorticoids, remains to be determined [79, 80]. Moreover, ambient levels of CORT taken during or immediately after stress exposure do not appear to be predictive of central IL-1 expression [63, 65]. Regardless, the point here is that the long-held assumption that glucocorticoids exert unilateral, anti-inflammatory actions does not broadly account for the literature writ large.

With that said, there are a variety of cellular mechanisms by which glucocorticoids impact gene expression for cytokines during times of stress, particularly in the case of IL-1 gene expression. Many of these effects are produced through interaction of the CORT-receptor complex with repressor sites in the promoter region of the IL-1 gene, including the nGRE repressor site and interference with nuclear factor κ-light chain-enhancer of activated B cells (NFkB) signaling. In addition, CORT has been shown to block cAMP response element-binding (CREB) phosphorylation, prevent NFkB binding to the DNA, and destabilize mRNA for IL-1 [81–83]. As such, CORT can interfere with IL-1 gene expression (and expression of other cytokines) through numerous pathways.

When taken together, a simple framework for understanding the interaction between the major stress-responsive systems (SNS and HPA axis) and inflammatory consequences of stress is that neuroinflammatory consequences of stress are mechanistically intertwined between the stimulatory actions of the SNS and the inhibitory actions of CORT (see [84] for a review), though much work remains to be done in this area. This concept is depicted in Fig. 5.1, which illustrates the basic mechanisms and pathways that appear to be involved in IL-1 regulation by stress. Although not a primary focus here, it is important to note that others have established prostaglandins as another highly stress-responsive, proinflammatory mediator within the CNS. The conversion of arachidonic acid to a particular prostanoid occurs via a COX-2 dependent pathway during times of inflammation. Upregulation of COX-2 expression has been observed as a result of stressor exposure in several brain regions [85], as has increased activity of other components of the pathway responsible for prostaglandin synthesis [86]. Furthermore, it seems that NE and CORT appear to exert opposing changes in prostanoid synthesis, with NE having been shown to stimulate prostaglandin E2 (PGE2) and CORT conversely inhibiting PGE2 (see [87] for review). Taken together, these data suggest that the overarching mechanisms governing neuroinflammatory consequences of stress may generalize across multiple inflammatory signaling families.

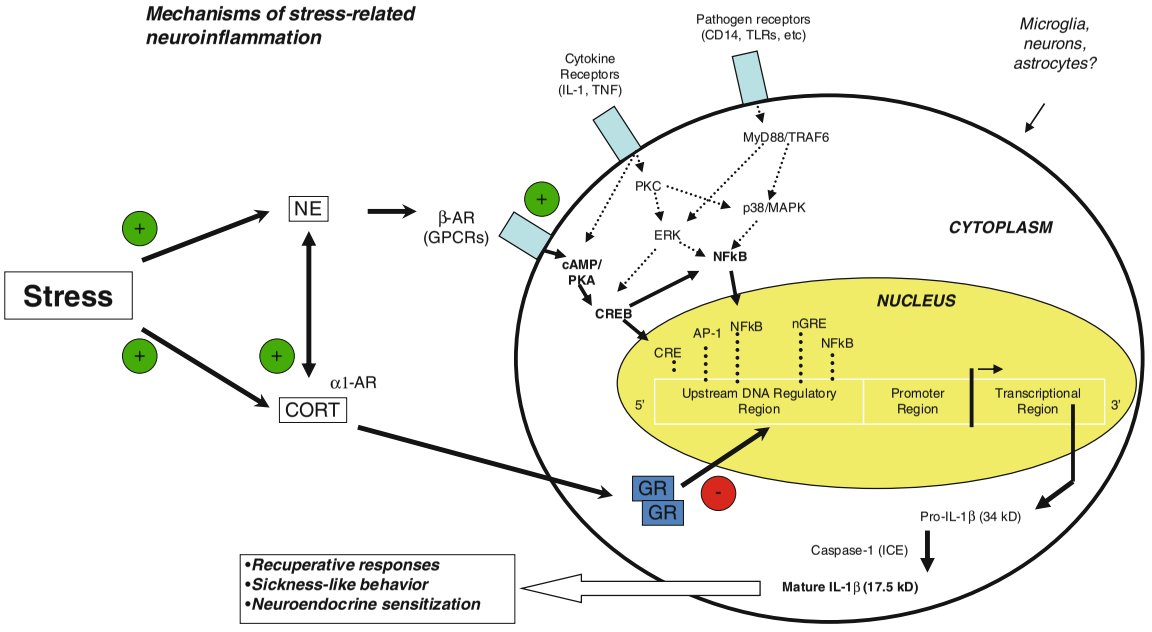

Fig. 5.1 Mechanisms of stress-related neuroinflammation. Exposure to stress challenges is associated with activation of both the sympathetic nervous system (SNS) and the hypothalamic–pituitary–adrenal (HPA) axis. These two principal stress-responsive systems involve the release of norepinephrine (NE) centrally and the release of corticosterone (CORT) from the adrenal glands. Results from mechanistic studies suggest that interaction of NE with β-adrenergic receptors leads to increased expression of the cytokine interleukin-1-beta (IL-1) in key regions of the CNS. Though the specific intracellular signaling pathways leading to IL-1 expression and the cell types expressing IL-1 have not yet been determined, evidence suggests that multiple signaling pathways and cell types may be involved in stress-dependent cytokine expression. CORT, on the other hand, appears to negatively influence the expression of IL-1 and other cytokines through interaction with glucocorticoid responsive elements (GREs) in the promoter region of cytokine genes. There are also reciprocal interactions between NE and CORT, whereby NE is a key driver in central regulation of the HPA axis (via α1-adrenergic receptors) and CORT tempers the release of NE. Ultimately, the release of mature IL-1 in the CNS has been linked to the expression of sickness-like behaviors, alteration of neuroendocrine processes including sensitization of the HPA axis, and other recuperative responses

Though the review above is largely centered on the role of NE as a key driver of inflammatory processes during times of stress, we would be remiss if we did not also point out alternative mechanisms that may be involved in regulation of neuroinflammation by stress. For instance, Nair and Bonneau [66] showed that the microglial proliferation associated with repeated stress was attenuated by administration of MK-801. Considering that glutamate plays an equally important role in the initiation of the stress response [88], and excitatory amino acids are directly coupled to inflammatory signaling pathways [86, 89, 90], it is not surprising that the general level of cellular excitation in the CNS microenvironment appears to be predictive of cytokine release [16, 91]. With that said, it is tempting to conclude that the relative state of neuronal excitation of a brain site under stressful conditions may show a strong association with IL-1 gene expression or the expression of other cytokines. However, our recent studies [61] (also Hueston et al. in prep) suggest that c-fos expression does not perfectly align with IL-1 expression but can serve as a useful comparator for understanding the relationship between cytokine expression and neuronal activation patterns more generally under certain conditions. In sum, the regulation of cytokine expression by stress is highly complex and probably involves a wide variety of ligands within the CNS, with a fair degree of convergence in signaling pathways known to be involved in cytokine gene expression.

In attempting to examine the stress-dependent changes in cytokines and the mechanisms responsible for these alterations, one of the major challenges associated with these studies has been the confounding impact of prior cranial surgery. This is largely to be expected due to the cellular trauma associated with implantation of guide cannula, microinjection of drugs, or insertion of injectors/electrodes to produce permanent lesions (e.g., [92]). However, designing studies without use of these conventional neuroscience techniques has proven to be quite difficult for several reasons. For instance, in microinjection studies, we have observed large and reliable increases in IL-1 protein expression after injection of sterile physiological saline into the third ventricle relative to noninjected (cannulated) controls despite agonizing attention to sterile process at all levels of the procedure. In fact, these changes are so large that they have masked our ability to detect increased IL-1 protein after injection of microgram quantities of LPS into the third ventricle (relative to vehicle-injected controls). The second, more subtle, problem associated with cranial surgery is how the surgical experience impacts reactivity to later stress challenges. We have other unpublished studies demonstrating marginal increases in basal IL-1 expression 10 days after cranial surgery in sites distal to the microinjection, which appeared to relegate subjects nonresponsive to the stress challenge. That is, rats receiving prior cranial surgery failed to show stress-dependent changes in cytokines, which have been highly reproducible in all studies except those involving cranial surgery. While intracisterna magna injections under brief anesthesia probably offer one viable alternative to cranial surgery, this procedure is also not without impact on central inflammatory processes and certainly impacts other more acute, labile stress measures [adrenocorticotropic hormone (ACTH), CORT, and behavioral indices of stress/anxiety].

With that said, these problems appear to be unique to situations where cranial surgery is performed and endogenous cytokine responses are being measured as

dependent variables. The use of cranial surgery to implant guide cannula for microinjection of compounds with anti-inflammatory properties to try and reverse some behavioral or neurochemical process that is expected to be driven by stress-dependent cytokines has been much more successful. While this may seem ironic at first, it should be noted that central injection of anti-inflammatory compounds would be expected to reduce inflammatory consequences evoked by the stress challenge as well as any ongoing inflammation evoked by cannula-associated damage and/or delivery of the vehicle as well. We raise these issues as a cautionary note to others who seek to pursue stress-dependent changes in cytokines and/or their behavioral consequences. Aside from closely monitoring sterile procedure, we recommend incorporation of ultimate controls (i.e., unoperated subjects) where possible to clarify the influence of invasive procedures on the outcomes of interest.

5.3 Ethanol and Neuroinflammation

5.3.1 Ethanol Exposure Alters Both Peripheral and Central Cytokine Expression

Research has consistently demonstrated that alcohol exposure is capable of inducing alterations in the expression of many cytokines across a variety of locations, including the plasma, liver, and brain (e.g., see [93]). Historically, the effects of alcohol on peripheral cytokine expression have been well investigated in terms of the role that immune signaling plays in liver and organ damage following long-term alcohol consumption in humans. Taken together, these types of studies have generally shown that in humans, following chronic heavy alcohol consumption, levels of cytokines such as TNF-α, IL-6, and IL-1 are significantly elevated in the plasma (for review, see Table 2 of [94]). More specifically, it has been hypothesized that initial alcohol-induced increases in cytokines leads to the activation of T helper cytokines, with increased T helper 1 (Th1) cytokines being associated with early liver damage (hepatitis) and a switch from expression of Th1 to Th2 cytokines related to more pronounced liver disease (fibrosis/cirrhosis) (see [93] for review).

The influence of alcohol exposure on peripheral cytokine expression has also received considerable attention in animal models, with the effects of alcohol exposure on central cytokine levels beginning to receive increasing research interest as well. The results from animal studies investigating alterations in peripheral and central cytokines following alcohol exposure as well as antigen-stimulated production of cytokines after alcohol administration are presented in Table 5.1. These studies have been organized according to location of cytokine change (i.e., central versus peripheral), species under investigation (rats versus mice), and type of alcohol exposure (acute versus chronic).

We will first consider alterations in peripheral cytokines, which have primarily been examined as a means of understanding the effects of ethanol on peripheral organ damage and increased susceptibility to infection. In most instances, these experiments have explored the effects of alcohol exposure on antigen-stimulated

Table 5.1 Studies investigating alcohol–cytokine interactions

Species	Ethanol exposure	Results	Reference
Central			
Rat	Acute (cultured astrocytes treated with 50–300 mM)	Acute exposure of rat cortical astrocyte culture to EtOH lowered TNF-α expression at 300 mM, while stimulating IL-6 secretion at 100 mM and above	Sarc et al. [95]
	Acute (cultured microglia stimulated with 10, 50, and 100 mM)	EtOH-stimulated cells exhibited increased release of both TNF-α and IL-1β at all the concentrations used at 7, 24, and 48 h, relative to baseline	Fernandez-Lizarbe et al. [96]
	Chronic (50–300 mM × 7 days)	• Chronic exposure of rat cortical astrocytes to 50, 100, or 200 mM EtOH significantly elevated IL-6, with IL-6 decreased at concentrations greater than 200 mM EtOH • TNF-α secretion was dose-dependently reduced by EtOH	Sarc et al. [95]
	Chronic (5 g/kg i.g. on 3 consecutive days)	EtOH exposure on PND 7–9 resulted in a significant increase in levels of TNF-α and IL-1β in both cerebral cortex and hippocampus compared with controls	Tiwari and Chopra [97]
	Chronic (10 weeks of once daily10 g/kg EtOH i.g.)	Elevated levels of TNF-α and IL-1β in hippocampus and cerebral cortex of EtOH-consuming rats compared to controls	Tiwari et al. [98]
	Chronic (4–6 weeks liquid EtOH diet)	Chronic EtOH diet led to an increase in TNF-α and IL-6 expression in the hypothalamus, pituitary, and ovary of female rats	Emanuele et al. [99]
	Chronic (5 mos of liquid diet exposure OR cultured astrocytes exposed to 75 mM EtOH for 7 days)	• Chronic EtOH exposure upregulated expression of IL-1β and COX-2 in the cortex of EtOH-fed rats relative to controls • Cultured astrocytes exposed to long-term EtOH also exhibited increased production of IL-1β and COX-2	Valles et al. [100]
	EtOH withdrawal (following chronic intermittent EtOH exposures)	Preexposure to systemic LPS, central IL-1β, or central TNF-α prior to chronic intermittent EtOH exposure resulted in sensitized withdrawal-induced anxiety	Breese et al. [101]

(continued)

Table 5.1 (continued)

Species	Ethanol exposure	Results	Reference
Mouse	Acute (cultured microglia stimulated with 10, 50, and 100 mM)	In wild-type mice, EtOH-stimulated cells exhibited increased release of TNF-α, whereas TLR-deficient mice did not show this EtOH-related increase	Fernandez-Lizarbe et al. [96]
	Acute (1 day 5 g/kg i.g.)	• Increased brain expression of TNF-α and MCP-1 mRNA by an acute EtOH exposure, with cytokine gene expression either decreased or unchanged in the liver • LPS-induced cytokine production was increased in EtOH challenged versus vehicle mice	Qin et al. [102]
	Acute (BEC of 100 mg/dl, i.p.)	Acute dose of EtOH increased levels of TNF-α, IL-1β, and IL-6 protein in the hypothalamus 48 h after exposure	Emanuele et al. [103]
	Acute (100 mM)	Exposure of BV-2 microglial cells to EtOH significantly decreased LPS-induced IL-1β release	Lee et al. [104]
	Chronic (5 months ETOH-containing water, 10%)	Chronic EtOH consumption upregulated TNF-α, IL-1β, and IL-6 mRNA expression in cortex in WT mice compared to pair-fed WT controls, with EtOH-related increases not observed in TLR4-knockout mice	Alfonso-Loeches et al. [105]
	Chronic (daily 5 g/kg EtOH, i.g., for 10 days)	Increased expression of TNF-α and MCP-1 mRNA from EtOH exposure alone was reported in brain, with no changes in LPS-induced cytokine expression observed in relation to the EtOH exposure	Qin et al. [102]

(continued)

Table 5.1 (continued)

Species	Ethanol exposure	Results	Reference
Peripheral			
Rat	Acute (7 h i.v. EtOH infusion after priming dose)	In rats administered EtOH, TNF-α secretion was generally reduced in alveolar macrophages challenged in vitro with LPS relative to control rats	D'Souza et al. [106]
	Acute (2 g/kg i.g.)	Acute EtOH challenged resulted in significantly attenuated endotoxin-induced TNF levels in serum compared to vehicle rats	Honchel et al. [107]
	Acute (BEC levels of 75–175 mg/dl)	Endotoxin-induced serum levels of TNF-α decreased dose-dependently by prior EtOH administration	D'Souza et al. [108]
	Acute	EtOH exposure attenuated LPS-induced production of TNF in serum and lung	Nelson et al. [109]
	Chronic (daily10 g/kg EtOH i.g. for 10 weeks)	Increased TNF-α and IL-1β in serum/sciatic nerve of EtOH-exposed rats relative to controls	Tiwari et al. [110]
	Chronic (Kupffer cells, 4 weeks of daily 5 g/kg EtOH i.g.)	Chronic EtOH exposure increased TNF-α production in Kupffer cells after LPS stimulation compared to pair-fed controls	Enomoto et al. [111]
	Chronic (20 weeks of EtOH liquid diet)	• EtOH diet increased levels of TNF-α, IL-1β, and IL-10 in serum • EtOH diet also further augmented LPS-induced increases in IL-10 and IL-1β	Valles et al. [112]
	Chronic (4 weeks of EtOH diet)	Long-term EtOH consumption increased TNF-α, and decreased IL-1β, production after LPS stimulation in Kupffer cells	Kishore et al. [113]
	Chronic (Kupffer cells of rats on liquid diet of 17–35% EtOH for 2+ days)	Chronic EtOH exposure increased expression of TNF-α following LPS stimulation	Kishore et al. [114]
	Chronic (12–14 weeks liquid EtOH diet)	Both spontaneous and LPS-stimulated TNF-α secretion was generally reduced in alveolar macrophages of rats consuming EtOH, relative to pair-fed control rats	D'Souza et al. [106]
	Chronic (6 weeks liquid diet)	Long-term EtOH consumption resulted in exacerbated TNF serum levels in response to an endotoxin challenge, relative to pair-fed rats	Honchel et al. [107]

(continued)

Table 5.1 (continued)

Species	Ethanol exposure	Results	Reference
Mouse	Acute (6 g/kg i.g.)	In WT mice, levels of all cytokines measured (except for IL-10 and MIP-2) were decreased by previous EtOH exposure in response to *E. coli* with far fewer affected by EtOH in TLR4 mutant mice	Bhatty et al. [115]
	Acute (binge drinking model 4 or 6 g/kg i.g.)	Acute EtOH decreased immunogen-induced activation of proinflammatory cytokines for several hours after *E. coli* administration. In serum, at 21 h post-EtOH, IL-1β and IL-6 were increased in EtOH plus LPS mice	Pruett et al. [116]
	Acute (6 g/kg i.g.)	EtOH alone did not exact any changes on cytokines levels, while EtOH and poly I:C in combination suppressed serum TNF-α, but caused no changes in serum IL-1β, IL-6, or IL-10	Glover et al. [117]
	Acute (6 g/kg i.g.)	Acute EtOH (in vivo) delivered 30 min before LPS administration generally blocked production of proinflammatory cytokines by LPS	Pruett and Fan [118]
	Acute (cultured murine macrophages treated with either 10, 50, 100 mM EtOH)	• EtOH exposure alone generally increased release of TNF-α and IL-1β • EtOH in combination with LPS attenuated LPS-induced TNF-α production • EtOH exposure alone led to an increase release of IL-10	Fernandez-Lizarbe et al. [119]
	Acute (binge EtOH, i.g.)	Suppression of LPS-induced cytokine production in peritoneal fluid and serum	Dai and Pruett [120]
	Acute (3–6 g/kg i.g.)	Acute exposure suppressed LPS-induced serum IL-6 levels in a dose-dependent manner	Pruett and Pruett [121]
	Acute (treatment of macrophage cell culture line with 0.05–0.4% wt/v EtOH)	Acute EtOH exposure suppressed the LPS-induced increases in TNF-α levels	Dai et al. [122]
	Acute (2.9 g/kg i.p.)	Splenic macrophages isolated 3 h after EtOH exposure exhibited reduced IL-6 and TNF-α production in response to ligands on TLR4, TLR2, and TLR9	Goral and Kovacs [123]

(continued)

Table 5.1 (continued)

Species	Ethanol exposure	Results	Reference
	Acute (6 g/kg, i.g.)	In vivo, cytokines induced by either LPS or poly I:C were generally suppressed by EtOH (e.g., IL-6, IL-12), while IL-10 was increased, if changed at all. Results from the in vitro preparation where not the same as in the in vivo model	Pruett et al. [124]
	Acute (150 μl i.p.)	Splenic macrophages isolated for 3 or 24 h after EtOH exposure demonstrated decreased LPS-induced IL-6 production; effect disappeared after 48 h and no IL-6 changes in non-LPS-treated animals were observed	Goral et al. [125]
	Acute (6 g/kg i.g.)	Binge EtOH exposure suppressed poly I:C-induced inflammation in peritoneal macrophages (e.g., decreased IL-6 and IL-12 mRNA)	Pruett et al. [126]
	Acute (6 g/kg i.g.)	• Acute EtOH exposure generally suppressed the immune activating effects of several TLR ligands, although the exact cytokine/chemokine suppressed depended on serum versus peritoneal sample and ligand of interest • IL-10 production tended to be raised by EtOH exposure	Pruett et al. [127]
	Acute (4–7 g/kg i.g.)	Binge EtOH exposure decreased poly I:C-induced production of many cytokines in serum (TNF-α, IFN-β, IFN-γ, IL-6, IL-9, IL-12, and IL-15), yet increased poly I:C production of IL-10	Pruett et al. [128]
	Acute (4 g/kg i.p.)	EtOH exposure lowered LPS-stimulated IL-12 protein levels in blood and lung, but raised IL-10 production	Mason et al. [129]
	Acute (5–7 g/kg i.g.)	Binge EtOH exposure lowered *P. acnes*-induced TNF-α production in peritoneal samples	Vinson et al. [130]
	Acute (6 g/kg i.g.)	Acute EtOH exposure significantly decreased of IL-1β, IL-2, and IL-4 in response to SRBC challenge compared to non-EtOH-exposed controls	Han and Pruett [131]

(continued)

Table 5.1 (continued)

Species	Ethanol exposure	Results	Reference
	Chronic (daily 5 g/kg EtOH, i.g., for 10 days)	In liver, increased expression of TNF-α, MCP-1, IL-1β, and IL-10 mRNA was observed to the EtOH exposure alone, with LPS-induced increases in TNF-α exacerbated by repeated EtOH exposure	Qin et al. [102]
	Chronic (4 weeks EtOH-containing water, 20%)	Long-term exposure to EtOH resulted in general tolerance to EtOH-related suppression of LPS-induced cytokine production in serum and peritoneal fluid	Dai and Pruett [120]
	Chronic (7 weeks EtOH diet)	No changes were observed in liver cytokines from exposure to EtOH diet alone LPS-induced increases in cytokines were more pronounced in EtOH-fed mice compared to controls, particularly among IL-10 mutants	Hill et al. [132]
	Chronic (11 weeks of 5% EtOH liquid diet)	Slight but significant decrease in LPS- and concanavalin A-induced production of several cytokines in both splenocytes and thymocytes	Wang et al. [133]

Notes:
Numbers listed in brackets refer to reference number in bibliography
Abbreviations used: blood ethanol concentration (*BEC*); cycloxygenase-2 (*COX-2*); *Escherichia coli* (*E. coli*); ethanol (*EtOH*); interferon-beta (*IFN-β*); interferon-gamma (*IFN-γ*); interleukin-1 beta (*IL-1β*), interleukin-2 (*IL-2*); interleukin-4 (*IL-4*); interleukin-6 (*IL-6*); Interleukin-9 (*IL-9*); interleukin-10 (*IL-10*); interleukin-12 (*IL-12*); interleukin-15 (*IL-15*); intravenously (*i.v.)*; intragastrically (*i.g.*); intraperitoneally (*i.p.*); lipopolysaccharide (*LPS*); monocyte chemoattractant protein-1 (*MCP-1*); macrophage inflammatory protein-2 (*MIP-2*); *Propionibacterium acnes* (*P. acnes*); polyinosinic:polycytidylic acid (*poly I:C*); postnatal days (*PND*); toll-like receptor(s) (*TLR*); toll-like receptor-4 (*TLR-4*); tumor necrosis factor (*TNF*); tumor necrosis factor-alpha (*TNF-α*); sheep red blood cells (*SRBC*)

immune responses and in general have reported that alcohol tends to have an overall dampening effect. It is noteworthy to mention that this trend parallels what was observed in the stress-immune literature when it was in its infancy as well. For instance, in mice, it has been found that acute binge-like doses (4–7 g/kg) of ethanol delivered intragastrically (i.g.) are capable of attenuating antigen-stimulated production of several proinflammatory cytokines in both serum and peritoneal samples (e.g., see [120, 128, 130]). Similarly, studies with rats have also shown that acute in vivo alcohol exposure suppresses later stimulation of proinflammatory cytokines by immunogens in serum (e.g., [107, 109]) and in lung (e.g., [109]). One way by which acute alcohol might decrease the proinflammatory response is through its interaction with toll-like receptors (TLRs), which are bound by immune challenges such as LPS and polyriboinosinic polyribocytidylic acid (poly I:C). This mechanism is supported by the observation that these attenuations in immunogen-precipitated

proinflammatory cytokine production have been shown to depend upon the immunogen of interest. Specifically, ethanol has been shown to differentially impact the effects of ligands to the various TLR subtypes (i.e., LPS as a TLR-4 ligand; poly I:C as a TLR-3 ligand) (see [127]), with these effects also dependent upon the type of sample examined (i.e., serum versus peritoneal lavage versus alveolar sample). Additionally, though in vitro preparations also tend to show similar effects to the in vivo studies, differential effects of ethanol on antigen-stimulated cytokine production may also be observed in these two preparations (e.g., see [124]). In contrast to proinflammatory cytokines, in several instances, peripheral production of IL-10 (an anti-inflammatory cytokine) following immune challenge was shown to be *increased* after acute ethanol exposure in mice [124, 127, 128]. Together, a suppression of proinflammatory cytokines coupled with enhancement of anti-inflammatory cytokines, would lead to an immunosuppressive state, that is hypothesized to then act as a mechanism by which antigen exposure may lead to peripheral tissue damage.

While acute ethanol exposure has been shown to suppress peripheral production of proinflammatory cytokines after an immune challenge, in general, more chronic administration of ethanol has been reported to result in the opposite situation—an enhancement in production of these cytokines in response to an immunogen. In rats, long-term forced intake of ethanol via a liquid diet or ethanol-containing water source has been reported to increase levels of various proinflammatory cytokines in the periphery. For example, Valles et al. [112] reported increased IL-1β in the serum following LPS challenge in rats fed an ethanol-containing liquid diet for 20 weeks, compared to pair-fed rats. Similarly, only 6 weeks of exposure to an ethanol diet lead to enhanced endotoxin-induced increases in serum TNF-α levels in rats [107], while 4 weeks of daily ethanol (5 g/kg, i.g.) produced comparable results [111]. Parallel effects have also been observed using mice, with either 10 repeated days of ethanol gavage (5 g/kg) [102] or 7 weeks exposure to an ethanol-containing diet [132] exacerbating LPS-stimulated production of TNF-α in liver relative to vehicle-exposed controls, although these long-term effects of ethanol have not been ubiquitously observed (see [133]).

More recently, researchers have begun to investigate the impact of ethanol exposure on antigen-stimulated cytokine expression in the brain. In a recent study using mice, for instance, it was reported that acute ethanol exposure further enhanced the production of brain cytokines following a systemic LPS challenge [102]. In the same study, a more chronic regimen of ethanol exposure (5 g/kg i.g. for 10 days) was also found to potentiate levels of LPS-induced central cytokine production, with these increases lasting much longer in the brain than in the periphery. Additionally, it has been observed that microglial cells exposed to ethanol and then given LPS exhibited reduced levels of IL-1β relative to microglia not exposed to ethanol but challenged with LPS [104]. Clearly, more studies are needed in order to truly understand the effects that ethanol has on central production of cytokines in response to an immune challenge, with factors such as species, in vivo versus in vitro models, and dose influencing the results obtained.

Although an understanding of the involvement of cytokines in alcoholism-related tissue damage is of great importance, more recently researchers have begun to recognize the role that cytokines may play in the development of addictive processes

[93, 134, 135]. In particular, it is becoming apparent that alcohol exposure has the potential to alter peripheral or brain cytokines levels in the absence of an immune challenge, which may, in turn, impact behavioral responses to alcohol, stress-related processes [e.g., HPA axis; extrahypothalamic corticotropin-releasing hormone (CRH)], or physiological processes (e.g., SNS). Although the literature in this area is limited and has not always reported alcohol-induced changes in cytokines (e.g., see [117, 132]), there is mounting evidence to suggest that alcohol in and of itself is capable of significantly altering both peripheral and central cytokine expression. For example, Tiwari and colleagues have shown that chronic (10 weeks) exposure to ethanol induces increased levels of TNF-α and IL-1β in both the hippocampus and cortex in rats [98], as well as in the serum and sciatic nerve [110]. The same group also demonstrated that early postnatal exposure to ethanol on postnatal days (PND) seven to nine increased expression of TNF-α and IL-1β in the cortex and hippocampus [97]. Enhancement of cytokine expression in the serum [112] and cortex [100] following long-term ethanol intake has also reported by another group, and with mice as well [105]. Furthermore, when acute and chronic administration of ethanol was compared, Qin et al. [102] reported that both a single ethanol exposure, or ten repeated exposures to ethanol, resulted in increased levels of TNF-α and MCP-1 in brain, whereas liver expression of TNF-α, MCP-1, IL-1β, and IL-10 mRNA in liver was significantly decreased by ethanol administration.

In terms of stress-responsivity and immunological function, the hypothalamus is a brain region that is of great importance, particularly in the production of cytokines. When male mice were exposed to an acute systemic injection of ethanol, hypothalamic protein levels of TNF-α, IL-1β, and IL-6 were significantly increased 48 h later [103]. Similarly, when female rats were given long-term oral exposure to an ethanol diet, TNF-α and IL-6 levels were increased in both the hypothalamus and pituitary [99]. Indeed, recent data from our laboratory has shown that an acute binge dose of ethanol (4 g/kg) increased expression of IL-6 mRNA in several brain regions 3 h after exposure, including the PVN and hippocampus, whereas IL-1 mRNA levels were decreased, at this same time point (Deak et al. in prep). Recent in vitro work with both cultured astrocytes [95, 100] and microglia [119] has also demonstrated the capability of ethanol exposure alone to influence cytokine expression in brain, although the directionality of these effects seems to depend upon many factors, including cytokine of interest, dose of ethanol used, and duration of exposure.

Importantly, the timing of the assessment of cytokine changes in relation to ethanol exposure may be a key factor dictating the type of alterations observed in either peripheral or central inflammatory responses. Research that has focused specifically on the effects of withdrawal from ethanol administration on immune function has shown, for example, in response to a LPS challenge during alcohol withdrawal in humans monocyte derived cytokines, IL-1β, increased yet TNF-α and IL-12 decreased in controls. In another study [136], human alcoholics were shown to have decreased peripheral blood levels of CD4+ and CD8+ T cells during acute intoxication and withdrawal, as well as decreased levels of natural killer (NK) cells when compared to healthy controls, with, however, acute withdrawal increasing these subjects' blood monocyte levels. In contrast, Laso and colleagues [137] found increased peripheral blood levels of CD4+ T cells and NK cells during withdrawal

from chronic alcoholism, but this difference may be due to the length of time after drinking cessation, which was much longer in the latter study (9 months versus 1 week). In other studies, Riikonen and colleagues [138] observed an increase in cerebellar microglia following intermittent ethanol exposure, which they interpreted as an involvement of microglia in brain atrophy in alcoholism. Kim et al. [139] found that certain blood cytokine (IL-10, IL-12, IFN-γ) levels were increased in humans during hangover, while others [140] have found that serum levels of IL-8 are increased 36 h after acute ethanol administration. Cytokine expression in the blood, however, does not necessarily reflect what is occurring in the brain. For instance, while LPS-induced increases in serum TNF-α in rats were found to last less than a day, these increases persisted for at least 10 months in the brain [141]. Interestingly, in an animal model of withdrawal sensitization, it was also recently found that immune challenges may impact behavioral expression of ethanol withdrawal. When an immune challenge (LPS exposure) or direct application of a cytokine (IL-1B or TNF-α) was administered prior to repeated intermittent ethanol exposure, withdrawal-related anxiogenesis was sensitized in the rats that had previously received an immune challenge relative to controls [101].

5.3.2 Multiple Mechanisms May Contribute to the Production of Proinflammatory Cytokines in the CNS After Acute Ethanol Exposure

Proinflammatory cytokines were initially characterized for their role in communication between immune cells in the body and only in more recent years have been recognized as critical signaling molecules within the CNS. Since cytokines are produced directly by cells in the CNS as well as by immune cells in the body, there are at least three specific mechanisms by which acute ethanol administration might lead to increased expression of cytokines in the CNS. Although described as discrete mechanisms, these should not be regarded as mutually exclusive mechanisms by which acute ethanol influences central cytokines.

The first possibility is that ethanol may increase cytokines via direct actions on neurons or glia in the CNS. As a lipophilic compound, ethanol passes readily across the blood–brain barrier where it exerts its effects through direct actions on parenchyma of the CNS. In this regard, ethanol may directly activate cells in the CNS (neurons, microglia, or astrocytes) to produce IL-1 or other cytokines since each of these cell types participates in central cytokine production under varying conditions [16, 30], and active crosstalk between neurons and glia seems to promote adaptive CNS functioning [142]. Of particular relevance in the present context is the recent finding that acute ethanol exposure has been shown to activate microglia as evidenced by changes in reactive oxygen species in vitro [143]. Such telltale signs of microglial activation are often accompanied by increased cytokine production by microglia, suggesting these cells may be a potential source of cytokines during acute ethanol withdrawal. Central expression of IL-1 is also known to increase during excessive neural activity such as that seen during seizure activity [62, 144]. Considering that ethanol

withdrawal leads to increased neuronal activity that oftentimes approaches seizure levels (particularly when ethanol use/abuse is chronic [145]), it is quite possible that sustained high levels of neuronal activity produced during withdrawal from ethanol exposure might increase central cytokine expression.

Yet another possible mechanism for ethanol-associated alterations in cytokines may be through increased cytokine expression peripherally, thereby activating immune-to-brain communication pathways. For example, acute ethanol exposure has been shown to increase expression of proinflammatory cytokines (particularly IL-1 and IL-6) in the liver [112]. The liver is densely innervated by the vagus nerve, and the vagus is a well-established route of neural communication between the periphery and the CNS (e.g., [146]). In this scenario, proinflammatory cytokines produced and released in blood or organs bind to receptors on lymphoid tissue associated with vagal paraganglia, activate vagal transmission, and induce de novo synthesis of cytokines in projection regions of the vagus nerve, particularly in the NTS and hypothalamus [146]. At a functional level, electrical stimulation of the vagus nerve increases IL-1 expression in the hypothalamus [147], and subdiaphragmatic vagotomy blocks IL-1 responses in the hypothalamus produced by low doses of LPS or IL-1 injection [148, 149]. Similar effects have been observed with the sciatic [150] and glossopharyngeal [151, 152] nerves as well, suggesting that peripheral nerves may be common paths for immune-to-brain communication. A comparable mechanism might be proposed in the case of voluntary ethanol intake or during intragastric intubation where ethanol might act as an irritant to the GI tract (e.g., [153])—production of cytokines by intestinal epithelial cells may bind to receptors on peripheral nerves innervating the gut, thereby activating immune-to-brain signaling pathways and increasing central cytokine expression. In any case, these scenarios would indicate that central expression of cytokines during acute ethanol exposure and/or withdrawal could occur secondary to peripheral production of cytokines rather than due to a direct action of ethanol on the CNS parenchyma.

Finally, withdrawal from acute ethanol may increase cytokine production through an NE-dependent pathway. Acute ethanol withdrawal elicits robust activation of the HPA axis as well as the autonomic nervous system [154, 155]. Increased NE activity in the hypothalamus during withdrawal contributes to this HPA activation and may account for a variety of withdrawal-related changes in behavior [156]. Interestingly, increased NE release during withdrawal does not appear to be unique to alcohol, as withdrawal from chronic morphine has been shown to activate catecholamine-containing cells in brainstem autonomic nuclei, leading to NE release in the PVN and ultimately activation of the HPA axis [157]. Thus, there is considerable evidence to suggest that hypothalamic NE is increased during withdrawal from ethanol and other substances, ultimately contributing to withdrawal-related activation of HPA axis activity and expression of withdrawal-associated behaviors. As previously discussed (see above), NE has also been shown to be a powerful regulator of IL-1 expression, particularly in the hypothalamus. Therefore, it is a possibility that increased hypothalamic noradrenergic activity during ethanol withdrawal may induced expression of cytokines, providing a direct neurochemical-to-cytokine pathway by which acute ethanol withdrawal leads to increased cytokine expression. Indeed, this is an intriguing hypothesis that fits well with current theories of addiction/withdrawal [155].

5.3.3 *Reciprocal Interactions Between Alcohol Effects and Stress Challenges: Timing of Events and Levels of Analysis Yield Many Interesting Questions*

There is currently a rich literature investigating the interaction between stress and alcohol exposure. In particular, there has been a vast number of studies that have sought to elucidate the role that stressor exposure has on oral consumption of ethanol in both humans and in animal models (for review, see [158–161]). Although this is an especially important avenue of research, the influence of stressors on neural, hormonal, and behavioral responses to later ethanol access/exposure is also of importance. Of course, there is the potential for multiple (and not mutually exclusive) mechanisms to contribute to the effect of stressors on alcohol responsivity, with stress- and/or alcohol-induced alterations in central/peripheral cytokines just one of many possibilities.

Since studies examining alterations in cytokines following ethanol exposure alone (i.e., without immunological challenge) have, in fact, shown that ethanol is capable of altering central expression of some cytokines (see above), these alcohol-induced changes in cytokine levels may themselves contribute to changes in behavior during intoxication and/or withdrawal. In this sense, one might argue that cytokine changes observed during withdrawal could be the cause and/or consequence of distress produced by withdrawal, thereby raising interesting questions about whether anti-inflammatory agents might have "therapeutic" benefits for ameliorating the adverse consequences of ethanol withdrawal. If this is the case, then one might expect the pattern of cytokine expression to vary as a function of the phase of alcohol exposure (i.e., some cytokines might be elevated during intoxication, while others might relate more to alcohol withdrawal). Indeed, recent results from our laboratory have observed that both i.g. and intraperitoneal (i.p.) administration of ethanol changes cytokine expression in several brain regions. More specifically, a 4-g/kg challenge of ethanol resulted in increased expression of IL-6 mRNA in hypothalamus and hippocampus (as well as in the periphery) during peak intoxication, whereas IL-1 mRNA levels were decreased during intoxication and significantly increased during acute withdrawal from the ethanol exposure (Deak et al. in prep).

Based on these findings, one would predict that if cytokines are responsible for behavioral changes following ethanol exposure, then pharmacological studies should be able to uncover the mechanisms by which cytokines influence ethanol-induced behavioral alterations. More specifically, pharmacological blockade of cytokine signaling should be capable of attenuating behavioral consequences of ethanol exposure, either through more general suppression of cytokine actions or more specifically via antagonism of the action of particular cytokines. More recently, we have begun to explore this possibility by examining ethanol withdrawal-related behaviors. In animal models, acute ethanol withdrawal involves expression of a constellation of behavioral sequela, including hypoactivity, anxiogenesis, reduced social interactions, decreased food and water intake, and fever [162]. As described above, these

behavioral alterations closely resemble sickness behaviors expressed following immune challenge. The possibility remains, therefore, that induction of cytokines following exposure to ethanol may play a key role in the initiation of these behaviors. Current work from our laboratory [171] has begun to explore this possibility. Briefly, rats were given a large acute ethanol challenge (4 g/kg) in order to induce an acute withdrawal (or hangover) state [163–165], which was indexed as reduced exploration and social investigation in a modified social investigation task previously validated in our laboratory [59]. In two different experiments, we explored the ability of indomethacin or IL-1ra to reverse these acute withdrawal-associated behaviors, with these pharmacological treatments administered following the alcohol challenge, but prior to behavioral testing. Although administration of these anti-inflammatory substances did not successfully block expression of acute withdrawal-associated behaviors under these particular circumstances, these results do not preclude the involvement of cytokines in the expression of ethanol-related behaviors since several variables (such as dose of drugs used, timing of drug application relative to ethanol exposure, and the ability of drugs to distribute broadly throughout the CNS) could have impacted the results obtained.

Together, the studies described above provide some context for understanding how ethanol-induced cytokines might play a role in orchestration of ethanol-related behavioral changes. Another very interesting line of inquiry is how ethanol-related cytokine changes might impact the response to later stress challenge, or vice versa. In this regard, stress exposure before, during, or after intoxication (during acute withdrawal or beyond) may be significantly impacted by the individual's recent and/ or lifelong history of alcohol exposure. This is relevant because cytokines are an important mechanism by which the HPA axis becomes sensitized [7]. Furthermore, as stressors themselves (such as footshock) have been shown to increase cytokine expression [57, 58, 63], exposure to stress during intoxication or withdrawal could impact cytokine levels and feedback onto alcohol-induced alterations. Breese and colleagues have explored this possibility, for example, using a stress-sensitization model of ethanol withdrawal-related behaviors. Whereas a single 5-day exposure to an ethanol-containing diet did not result in acute withdrawal-induced anxiogenesis in a social interaction paradigm, this group has shown that repeated intermittent exposures to an alcohol diet did sensitize the withdrawal response and result in exacerbated anxiety during withdrawal [166]. Furthermore, repeated exposure to stress substituted for previous alcohol exposure, ultimately leading to significant withdrawal-related anxiogenesis after just one bout of ethanol diet exposure, which in and of itself, did not produce withdrawal-induced anxiety [167]. Recently, work from their laboratory examined cytokines as a mechanism underlying this stress sensitization of ethanol withdrawal behaviors, with repeated administrations of LPS or several other cytokines substituting for repeated stress or ethanol diet exposures in the expression of sensitization of withdrawal-related anxiety [101].

In Fig. 5.2, a potential framework for viewing the interactions between alcohol exposure, stress-related processes and inflammatory systems has been presented. Within our own laboratory, we have begun to explore the possible interaction

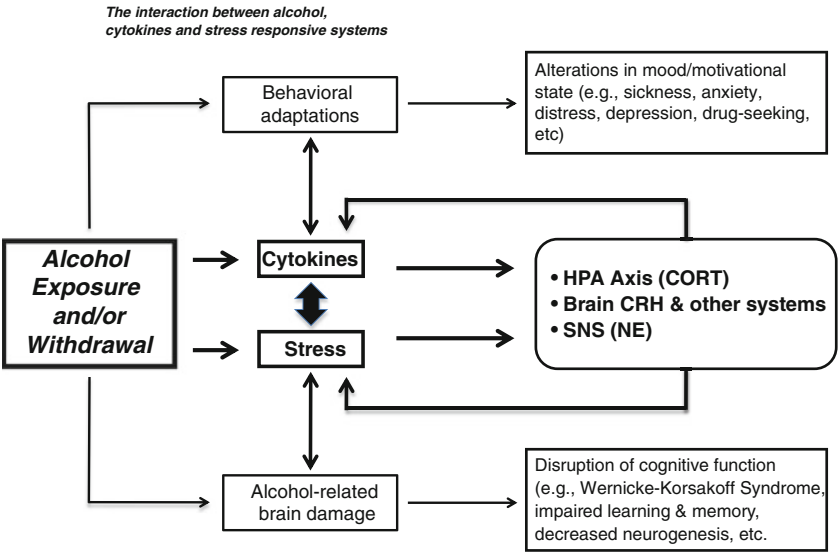

Fig. 5.2 The interaction between alcohol, cytokines, and stress-responsive systems. Exposure to and/or withdrawal from alcohol has the capacity to directly impact cytokine expression and is also associated with activation of stress-responsive systems. Given the powerful influence that cytokines exert over behavioral processes (e.g., sickness behaviors), it is likely that alcohol-induced changes in central cytokines play a key role in mediating alcohol-related behavioral adaptations. A growing body of research points toward long-term elevations in cytokines as a result of chronic alcohol use and abuse, which serve as a harbinger of alcohol-related brain damage. Given the cyclic interactions between alcohol exposure, cytokines, and stress-responsive systems, future studies delineating more precise mechanisms of central cytokine regulation by alcohol may hold promise for preventing the adverse consequences of long-term alcohol exposure

between stress, alcohol, and cytokines by investigating the HPA axis response to stress during withdrawal from an acute ethanol challenge since previous work has shown enhanced HPA activation to stressors during ethanol withdrawal [112, 168] (but see also [169]). Furthermore, we have been interested in whether any possible stress-related alterations in HPA activation are related to cytokine responses evoked by the stress and/or ethanol exposure itself. Briefly, we have recently reported that moderate acute stressors (e.g., restraint stress or exposure to a novel environment) imposed on adult male rats during peak withdrawal from an acute ethanol challenge were shown to significantly exacerbate stress-induced increases in plasma CORT [170]. While this initial work observed a stress hyperresponsive state during acute withdrawal, at this time, these changes have not been directly linked to ethanol-induced alterations in the expression of cytokines following stressor exposure [170]. Of course, the timing between ethanol exposure, stress exposure, and measurement of cytokines is certainly of importance and therefore does not necessarily rule out the involvement of cytokines in ethanol-withdrawal-related stress hyperresponsivity. Future studies in our lab will continue to explore these possibilities.

5.4 Conclusions and Future Directions

Our overarching goal here was to provide a broad overview of mechanisms and tribulations surrounding examination of stress-related inflammatory processes as well as the fledging literature surrounding alcohol–cytokine interactions. The central message we hoped to convey is that, to fully understand the interaction between alcohol and cytokines in brain and their potential implications for alcohol-related brain pathology, one must take into account the multitude of integrative, system-level interactions that are impacted by alcohol exposure, including, but not limited to, stress-responsive systems, liver–gut interactions, and the presence/absence of associated immunogenic stimuli (bacteria, viruses, etc.), as each of these will be determinants of cytokine expression in brain. Moreover, we assert that there is a wide body of literature now indicating a role for cytokines and other inflammatory processes in mediating the deleterious consequences of long-term alcohol exposure, whereas there is a relative paucity of studies seeking to identify early-term (acute), dynamic changes in cytokines in relation to alcohol, as well as how such effects might transform over the course of developmental history of the subject or in response to a growing number of alcohol exposures across the lifetime. Such studies will be instrumental in forging a near-certain link between initial alcohol exposure and the ultimate, adverse consequences of end-stage alcoholism.

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