HHS Public Access

Author manuscript

Alcohol Clin Exp Res. Author manuscript; available in PMC 2022 October 01.

Published in final edited form as:

Alcohol Clin Exp Res. 2021 October ; 45(10): 1908–1926. doi:10.1111/acer.14694.

Primed for addiction: A critical review of the role of microglia in the neurodevelopmental consequences of adolescent alcohol drinking

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Abstract

Alcohol is one of the most widely used recreational substances worldwide, with drinking frequently initiated during adolescence. The developmental state of the adolescent brain makes it vulnerable to initiating alcohol use, often in high doses, and particularly susceptible to alcohol-induced brain changes. Microglia, the brain parenchymal macrophages, have been implicated in mediating some of these effects, though the role that these cells play in the progression from alcohol drinking to dependence remains unclear. Microglia are uniquely positioned to sense and respond to central nervous system insult, and are now understood to exhibit innate immune memory, or "priming," altering their future functional responses based on prior exposures. In alcohol use disorders (AUDs), the role of microglia is debated. Whereas microglial activation can be pathogenic, contributing to neuroinflammation, tissue damage, and behavioral changes, or protective, it can also engage protective functions, providing support and mediating the resolution of damage. Understanding the role of microglia in adolescent AUDs is complicated by the fact that microglia are thought to be involved in developmental processes such as synaptic refinement and myelination, which underlie the functional maturation of multiple brain systems in adolescence. Thus, the role microglia play in the impact of alcohol use in adolescence is likely multifaceted. Long-term sequelae may be due to a failure to recover from EtOH-induced tissue damage, altered neurodevelopmental trajectories, and/or persistent changes to microglial responsivity and function. Here, we review critically the literature surrounding the effects of alcohol on microglia in models of adolescent alcohol misuse. We attempt to disentangle what is known about microglia from other neuroimmune effectors, to which we apply recent discoveries on the role of microglia in development and plasticity. Considered altogether, these studies challenge assumptions that proinflammatory microglia drive addiction. Alcohol priming microglia and there by perturbing their homeostatic roles in neurodevelopment, especially during critical periods of plasticity such

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

as adolescence, may have more serious implications for the neuropathogenesis of AUDs in adolescents.

Keywords

adolescent; alcohol; EtOH; microglia; neuroimmune

INTRODUCTION

Alcohol remains the most commonly used drug among adolescents in the United States where drinking rates in the Americas are the second highest in the world at 38.2% (Johnston et al., 2020; SAMHSA, 2018; World Health Organization, 2018). Adolescents (10 to 19 years, or 25 for some measures) drink less frequently than adults but consume similar quantities when they do drink (Deas et al., 2000; SAMHSA, 2018). Often, alcohol is consumed in "binges" defined as 4+ (females) or 5+ (males) standard drinks consumed within 2 h and achieving a blood alcohol level (BAL) topping 0.08% (>80 mg/dl; NIAAA, 2004). In 2018, 4.7% of adolescents aged 12–17 and 34.9% aged 18–25 were current binge drinkers in the United States (SAMHSA, 2019), while rates in Europe were even higher (Chung et al., 2018). Strikingly, approximately 10% of adolescents report extreme binge drinking of 10+ drinks per occasion with 5% reporting over 15 drinks in the past 2 weeks alone (Johnston et al., 2020; Patrick et al., 2013). Males remain overrepresented in the extreme binge drinking category (Patrick & Terry-McElrath, 2019), though the gender gap is narrowing in adolescents (Johnston et al., 2020).

Unfortunately, these surveys likely underestimate binge drinking in adolescents due to the definition of a binge, which is based on BALs in adults (NIAAA, 2004), and that teens underestimate the amount of alcohol in a standard drink (White et al., 2005). The smaller size and body weight of adolescents and therefore higher BAL per standard drink force reconsideration of these definitions for adolescents as they may hit the >0.08 mg% threshold with as little as 1–2 drinks within a 2-h period (Chung et al., 2018; Donovan, 2009). Such hazardous drinking patterns increase the risk for developing an alcohol use disorder (AUD), for which 1.6% of young adolescents aged 12–17 meet criteria, while older adolescents are similar to adult rates (~14%; Clark et al., 2002, 2016; Grant et al., 2015; SAMHSA, 2019). Adolescent drinking also predicts later alcohol misuse and/or dependence in adulthood (DeWit et al., 2000; Grant & Dawson, 1997).

Disentangling the factors that underlie this vulnerability toward excessive alcohol drinking in adulthood from those that are a consequence of adolescent drinking remains a challenge (Crum & Hulvershorn, 2020). Microglia have emerged as an interesting target due to not only their role in brain development and homeostasis but also their dual roles in response to insult both proinflammatory and reparative/resolving of inflammation. This critical review gathers and integrates findings on the effect of adolescent alcohol exposure on microglia and considers them in relation to processes that occur during adolescence and those that underlie vulnerability for addiction. Microglial activation states are outlined, and the concept of priming, a type of innate immune memory, is introduced. Finally, we highlight evidence

of microglial priming following adolescent alcohol exposure and identify several gaps in our understanding of the role of microglia and their activation in adolescent susceptibility to the causes and consequences of AUD pathogenesis.

ADOLESCENT VULNERABILITY FOR EXCESS ALCOHOL CONSUMPTION

Adolescence, typically 10 to 19 years of age, is a critical time for brain and behavioral maturation with neurodevelopmental changes occurring up to age 25 (Brenhouse & Schwarz, 2016; Lees et al., 2020; Spear, 2000). In rodents, adolescence ranges between postnatal days (PND) 28 and 42, with 43 to 55 considered late adolescence, especially in males (Brenhouse & Andersen, 2011; Spear & Brake, 1983). During this time, white matter volume increases remarkably from ongoing myelination, while gray matter volume decreases due to synaptic pruning and refinement (Giedd, 2004; Lees et al., 2020; Pfefferbaum et al., 2018). The relative developmental immaturity of the adolescent brain and behavior coupled with specific effects of alcohol in adolescents favor the development of alcohol misuse (Crews & Boettiger, 2009; Crews et al., 2016; Spear, 2000, 2018). Exploration, risk-taking, and sensation seeking are behaviors associated with adolescence and correspond to relatively immature regions and circuits in the adolescent brain (Spear, 2018). The brain's “go system,” including structures such as the mesolimbic nucleus accumbens (NAc) that are involved in reward and motivation, are functionally more mature at this time, whereas the “stop system” inclusive of the prefrontal cortices, involved in judgment and reasoning, develops later into adolescence (Gulley & Juraska, 2013; Markham et al., 2007; Sowell et al., 1999; Spear, 2018). Thus, age-typical impulsive behavior during adolescence likely results from hyperresponsivity to reward-based stimuli (Spear & Varlinskaya, 2005), while the underdeveloped top-down control systems hinder the ability of the frontal cortex to assess risk or exhibit impulse control (Gulley & Juraska, 2013; Townshend & Duka, 2005). Impulsive behavior, a hallmark of adolescence, often manifests as risk-taking or sensation seeking (Romer & Moreno, 2017), which are thought to play major roles in the initiation of alcohol use (Bardo et al., 2013; Crum & Hulvershorn, 2020; Hamilton et al., 2019). In preclinical models, measures of impulsivity are predictive of both increased sensitivity to drug reward (Yates et al., 2012) and dose-related aspects of subjective reinforcement (Marusich & Bardo, 2009).

In addition to an immature “stop system,” adolescents are more prone to excess consumption. Adolescents demonstrate enhanced sensitivity to the positive reinforcing action of alcohol with reduced sensitivity to sedation and motor impairment that normally help to regulate consumption (Gee et al., 2018; Li et al., 2003; Little et al., 1996; Spear, 2011, 2018). For example, rats consume more saccharin-sweetened ethanol (EtOH) solution during adolescence compared with adulthood, while consumption induced a greater tachycardic effect, which is associated with enhanced EtOH reward (Doremus et al., 2005; Ristuccia & Spear, 2008; Spear & Varlinskaya, 2005). Enhanced sensitivity to reward may be due to changes in the striatal dopaminergic system, which demonstrates high plasticity during adolescence, with evidence of increased activity and responsivity of mesolimbic and mesocortical projections (Gulley & Juraska, 2013; Lees et al., 2020; Silverman et al., 2015; Sowell et al., 1999; Spear, 2018; Wahlstrom et al., 2010). Indeed, bidirectional, dopaminergic projections between prefrontal cortex (PFC) and hippocampus - regions

important for modulating reward processing, attention, working memory, and episodic memory - are still maturing (Gee et al., 2018). Developmental differences in alcohol's effects on GABAergic neurotransmission may underlie the reduced sensitivity of adolescents to regulators of consumption, including the sedative/motor impairing effects of alcohol, compared with adults (Fleming et al., 2007; Li et al., 2003; Little et al., 1996). Several animal model studies, however, suggest faster EtOH metabolism in adolescents, which would also influence the sedative and motor impairing effects of EtOH (Walker & Ehlers, 2009; Watson et al., 2020). The literature varies widely, discussed below, and it remains a significant gap as to whether EtOH pharmacokinetic differences occur in human adolescents versus adults as well.

ADOLESCENT VULNERABILITY TO NEGATIVE CONSEQUENCES OF EXCESS CONSUMPTION

The adolescent's enhanced vulnerability to alcohol-induced neurotoxicity, particularly in regions still maturing, profoundly impacts development (Crews et al., 2000, 2016; Spear, 2018). These damaging effects occur within 2 domains: (1) alterations in brain structure/function through damage and/or derangement of physiology and (2) disruptions of the brain's developmental trajectory. Negative impact from either domain may persist into adulthood and drive and/or be exacerbated by continued alcohol misuse.

Imaging studies have revealed that immature regions such as the PFC are targets of alcohol toxicity (Gulley & Juraska, 2013; Jacobus & Tapert, 2013; Luciana et al., 2013; Pfefferbaum et al., 2018; Silveri et al., 2016; Squeglia et al., 2014). Seminal cross-sectional studies have shown that heavy drinking adolescents have smaller frontal and temporal gray and white matter volumes than low to no drinking teens (Alba-Ferrara et al., 2016; De Bellis et al., 2000, 2005; Fein et al., 2013; Jones et al., 2018; Medina et al., 2008; Silveri et al., 2016). However, the cross-sectional designs of this early work are marred by the limitations that predisposition (e.g., family history of AUD or psychiatric disorders) or existing structural deficits cannot be ruled out. Thus, results from preclinical models coupled with recent human, longitudinal data have been essential to our understanding of how the course of adolescent brain development is impacted by heavy drinking (Luciana et al., 2013; Pfefferbaum et al., 2018; Squeglia et al., 2014, 2015). Specifically, age-typical decreases in cortical gray matter volume were exacerbated, while expected increases in white matter volume were attenuated in heavy drinking adolescents (Luciana et al., 2013; Pfefferbaum et al., 2018; Squeglia et al., 2014, 2015). In addition, subcortical regions involved in the development of AUDs such as the hippocampus and amygdala also show volume reductions in adolescents with an AUD (De Bellis et al., 2000, 2005; Nagel et al., 2005). Preclinical studies are consistent with many of these deficits described in human adolescents as rodent models show that alcohol exposure leads to death of neurons and glia across cortical regions and hippocampus (Crews et al., 2000, 2016, 2019; Hu et al., 2020; Marshall et al., 2020; Morris et al., 2010a; Risher et al., 2015; Swartzwelder et al., 2019).

The increased risk for development of alcohol dependence or AUDs in adulthood after adolescent alcohol consumption is associated with long-term damage or impact on the

developmental trajectory of brain structures essential for normal, adaptive behaviors (DeWit et al., 2000; Grant & Dawson, 1997). However, it is difficult to extricate factors that underlie the propensity to drink to excess from the consequences of excessive alcohol drinking. Although alcohol-induced damage and neuroimmune effects in the adolescent are more widespread, we focus on 3 brain areas or circuits involved in addiction processes that are also vulnerable to alcohol damage and neuroimmune effects: the PFC, mesolimbic reward system, and hippocampus. Evidence from human imaging, behavior, and preclinical models converges for these 3 areas, though we acknowledge that this overlooks exciting new discoveries in regions underappreciated for their role in addiction, including especially, the cerebellum (Sullivan et al., 2020). We briefly review each area as a foundation for understanding the effects of innate immune system dysregulation by alcohol on these key regions that contribute to the pathogenesis of AUDs in adolescence.

Dysregulation of prefrontal cortical behavioral control systems is involved as both cause and consequence of adolescent alcohol drinking. Impaired behavioral control, or impulsivity, contributes to exploratory alcohol use during adolescence (Fernie et al., 2013); however, it also *results* from excess alcohol use and therefore plays a role in the persistence of this maladaptive behavior (Ivanov et al., 2021; Lopez-Caneda et al., 2014). Impulsivity or risky choice behavior has been observed in human heavy drinkers and preclinical models of AUD (Sanchez-Roige, Baro, et al., 2014; Sanchez-Roige, Pena-Oliver, et al., 2014). For example, emerging adult binge drinkers (aged 18 to 30) displayed greater motor impulsivity in a visual search matching task than nonbinge drinkers (Townshend & Duka, 2005). In adolescent males with higher self- and parental-reported impulsivity ratings, increased impulsive behavior was found to be a consequence of heavy drinking, an effect not seen in individuals with low a priori impulsive behavior ratings (White et al., 2011). Additionally, baseline measures of impulsivity in adolescents during the monetary incentive delay task were predictive of subsequent drinking rates, while heavy drinking predicted increases in impulsivity upon follow-up (Ivanov et al., 2021). In this cohort, blunted activity of the medial orbitofrontal cortex (OFC) in response to reward at baseline also predicted greater alcohol use (Ivanov et al., 2021). These behavioral studies parallel preclinical evidence that alcohol damages behavioral control systems, including the OFC required for response inhibition (Spinella, 2004), or alters neurotransmitter systems such as serotonin, dopamine, and acetylcholine, which are associated with heightened impulsivity or increased risky choice (Boutros et al., 2015; Leamy et al., 2016). For example, rats with the highest voluntary drinking in adolescence displayed greater risk preference in a probability discounting task (Nasrallah et al., 2009), while chronic adolescent alcohol exposure results in more prevalent risky choice in probability discounting tasks measured in adulthood, whether voluntary (Nasrallah et al., 2009; Schindler et al., 2014), or forced exposure to alcohol was used (Boutros et al., 2015). Rodent studies also implicate the OFC in both impulsive action and choice, where decreased OFC DAT function was associated with enhanced impulsive action (Yates et al., 2016), while enhanced OFC SERT function was associated with greater impulsive choice (Darna et al., 2015).

While the development of “top-down” control of the mesolimbic reward system emerges during adolescence, alcohol exposure during this time can derange the reward system, leading to an increased incidence of addictive-like behaviors in adulthood. For example,

in rats, 2-bottle choice alcohol consumption during adolescence increased appetitive and consummatory operant behaviors toward alcohol as adults (Amodeo et al., 2017). Interestingly, compared with controls, rats exposed to intermittent EtOH in adolescence displayed reduced frontolimbic functional connectivity following acute EtOH administration as adults, and showed decreased baseline resting-state connectivity between subregions of the PFC and between the PFC and striatum (Broadwater et al., 2018). Mechanistically, evidence from both animal and human studies supports that adolescent alcohol exposure persistently changes brain dopaminergic systems that may lead to enhanced vulnerability to developing an AUD in adulthood. Whole-cell patch clamp recordings in PFC slices from adult rats exposed to intermittent EtOH vapor during adolescence display a loss of D1 receptor modulation over intrinsic excitability and synaptic transmission between the mPFC and NAc core, and the mPFC and basolateral amygdala (Trantham-Davidson et al., 2017).

In rats, basal dopamine levels in the NAc septi reach adult levels by PND 35 and peak in late adolescence (PND 45) before returning to adult levels around PND 60 with levels of the dopamine metabolite, DOPAC, consistent from PND 35 forward (Philpot et al., 2009). However, there is a developmental transition in the rat's ability to adapt to repeated EtOH exposure between PND 35 and 45: Peak dopamine release upon acute EtOH challenge is blunted in younger (PND 25 and 35) versus older (PND 45 and 60) rats with a history of prior EtOH exposure. These data may reflect an enhanced susceptibility for addiction vulnerability during early adolescence (Philpot et al., 2009). Rats exposed intermittently to alcohol during adolescence consumed more alcohol in a 2-bottle choice test, which was driven potentially by enhanced motivation for EtOH in a self-administration paradigm, effects that were correlated with reduced c-Fos reactivity in the NAc (Alaux-Cantin et al., 2013). Alcohol-induced disruptions in dopamine signaling come about through a variety of mechanisms (Nixon & McClain, 2010). In humans, young adults who were either high reward-seekers, behaviorally disinhibited, or low responders to the effects of alcohol showed increased dopamine release in the striatum post oral alcohol administration versus placebo (Setiawan et al., 2014). Even moderate amounts of alcohol in adolescence enhanced a reward predictive cue through increasing phasic dopamine release during Pavlovian conditioned approach in adulthood (Spoelder et al., 2015). Polymorphisms in the D1 receptor have been associated with changes in signaling (BOLD response) in the medial OFC after an alcohol reward anticipation cue (Baker et al., 2019). Other neurotransmitter systems affected directly by alcohol also influence dopaminergic signaling. For example, increased GABAergic transmission in the ventral tegmental area leads to decreased basal dopamine, an effect thought to underlie maladaptive decision making as a consequence of adolescent alcohol drinking (Schindler et al., 2016).

Finally, hippocampal systems, which are well known for their canonical role in learning and memory, also contribute to behavioral control (Abela et al., 2013; Nixon et al., 2011; Rubin et al., 2017). Although this region has the least direct role in controlling consumption, it is a primary target of alcohol toxicity during adolescence, impacting PFC and mesolimbic functions that rely on interaction with the hippocampus (White & Swartzwelder, 2004). Adolescent binge drinkers consistently demonstrate learning and memory deficits across a variety of hippocampal-dependent tasks (Jacobus & Tapert, 2013; Mahmood et al., 2010; Schweinsburg et al., 2010; Vinader-Caerols, Duque, et al., 2017; Vinader-Caerols, Talk, et

al., 2017). These findings in humans are paralleled by several studies in preclinical models where alcohol exposure impairs learning and memory through accelerated forgetting, decreased retention, or other impairments on hippocampal-dependent tasks such as the Morris water maze or radial arm maze (Ji et al., 2018; Schulteis et al., 2008; Seemiller & Gould, 2020; Sircar et al., 2009; Sircar & Sircar, 2005; Swartzwelder et al., 2014). Indeed, impairments on the Morris water maze persisted longer in adolescent than in adult rats (Sircar & Sircar, 2005), which may reflect alcohol's more damaging effects on limbic circuitry in adolescents (Broadwater et al., 2014; Crews et al., 2000; Markwiese et al., 1998; Morris et al., 2010a). Following binge treatment, adolescent rats also displayed greater sensitivity to EtOH's memory impairing effects in a radial arm maze (Risher et al., 2013, 2015; White et al., 2000). For hippocampus, the role of adult neurogenesis must also be considered. Neurogenesis occurs throughout the lifetime of the organism, and alcohol impacts multiple aspects of this process, particularly in adolescence (Broadwater et al., 2014; Crews et al., 2006; Macht et al., 2019; McClain et al., 2014; Morris et al., 2010a; Sakharkar et al., 2016; Vetreno & Crews, 2015; reviewed in Wooden et al., 2021). Thus, both cell death and cell birth mechanisms contribute to the enhanced sensitivity of the adolescent brain to alcohol's damaging effects.

MECHANISMS OF ADOLESCENT VULNERABILITY TO ALCOHOL: MICROGLIA

Activation of the innate immune system is implicated in the causes and consequences of adult AUD (Crews et al., 2017; Erickson et al., 2019; Melbourne et al., 2019) but much less is known for adolescence. Growing evidence supports that innate immune system activation impacts adolescent vulnerability to developing AUDs at multiple levels, from influencing cell signaling to roles in synaptic and structural plasticity both during normal development and in response to insult. This review focuses on microglia, the hallmark cell of the brain's innate immune system. An overview of microglial functions in development and homeostasis is described in Figure 1A.

MICROGLIAL RESPONSE TO INSULT: PHENOTYPES AND PRIMING

Microglia were originally investigated in the context of illness and injury, where tissue examined histologically was noted to contain “reactive” microglia with enlarged cell bodies and retracted processes (Prinz et al., 2019). This marked response is now understood to reflect an extreme phenotype along a continuum of states of cellular activation (see Figure 1B for an overview of microglial activation; Dubbelaar et al., 2018; Kreutzberg, 1996). The microglial response to a particular stimulus appears to be highly dependent on the history of that cell's previous systemic or microenvironmental exposures, further complicating the picture. When sensitized, macrophages and microglia are referred to as primed, essentially a form of innate immune memory (Haley et al., 2018; Locati et al., 2013; Neher & Cunningham, 2019; Perry & Holmes, 2014; Ransohoff & Perry, 2009; Wendeln et al., 2018). Primed microglia, according to models of aging, stress, and neurodegeneration, do not exhibit a fully proinflammatory phenotype; they show an exaggerated or heightened response to an inflammatory stimulus compared with that observed in stimulus-naïve

microglia (Fonken et al., 2016; Neher & Cunningham, 2019; Perry & Holmes, 2014; Rosczyk et al., 2008). Identifying primed microglia remains challenging as unique markers have not been identified, though changes in morphology and up-regulation of cell surface antigens occurs (Perry & Holmes, 2014).

Numerous endogenous and exogenous stimuli have been shown to prime microglia. Of particular relevance to AUDs are pathogen-associated molecular patterns and endogenous damage-associated molecular patterns (DAMPs), or sterile insult signals that are released from neurons during cellular stress or neurotoxicity and are detected by microglial pattern recognition receptors (Haley et al., 2018). Molecules expressed in the degenerating or injured brain, such as colony-stimulating factor (Chapoval et al., 1998) and C-C motif chemokine ligand 2/monocyte chemotactic protein 1 (CCL2/MCP-1; Rankine et al., 2006), also prime microglia. The DAMP, high mobility group box protein 1 (HMGB1), has been implicated as a key factor mediating priming in stress, aging, and most recently in substance misuse including adolescent susceptibility to developing an AUD (Coleman et al., 2018; Crews et al., 2013; Kang et al., 2014; Wang et al., 2015; Weber et al., 2015; Yang & Tracey, 2010). Though the exact mechanisms of microglial priming remain poorly understood, evidence suggests that shifts in the microglial epigenetic landscape underlie these changes to microglial responsivity (Martins-Ferreira et al., 2020).

The priming phenomenon is pertinent to adolescence and AUD development as microglia are long-lived compared with their peripheral counterparts and therefore have a greater propensity to “remember” prior insults (Eyo & Wu, 2019; Neher & Cunningham, 2019). Activation of previously primed microglia results in a stronger inflammatory response and may exacerbate damage or disease progression. Evidence of priming in alcohol studies has been observed in repeated binge models in adult rats where a sensitized microglial response was observed (Marshall et al., 2016) or in a fetal alcohol spectrum disorder model (Chastain et al., 2019). The shift in cellular phenotype that occurs in primed microglia leads to a decreased expression of homeostatic genes. Studies in primed peripheral macrophages demonstrate an accumulation of permissive histone modifications at proinflammatory gene promoters and repressive modifications at genes that underlie homeostatic and reparative functions (Kang et al., 2017). Therefore, priming in adolescence may result in microglia that are redirected from their homeostatic, protective, and developmental functions. This persistent shift in microglial set point following perturbation provides a framework for understanding how cumulative insults, particularly those experienced during developmentally sensitive windows, could have a lasting impact on brain function later in life.

THE ROLE OF MICROGLIA IN NEURODEVELOPMENT

Across different stages of life, from embryogenesis to adolescence and adulthood, microglia display distinct morphologies, transcrip tomic and proteomic signatures (Masuda et al., 2019; Prinz et al., 2019). Though research demonstrates a critical role of these cells in sculpting early brain development, far less is known about their function in adolescence. As discussed above, the brain continues to mature throughout adolescence, with widespread fine-tuning of synaptic connections, and ongoing myelination and neurogenesis, all

processes in which microglia are thought to play an important role. Factors that affect the immune system during adolescence not only impact existing circuity but also perturb developmental trajectories, affecting development of brain circuits and behaviors, such as decision making, impulsivity and cognition, that occur during this time.

In the early developing brain, microglia induce normal programmed cell death, phagocytose dying neurons, and contribute to synapse formation and elimination, shaping the architecture and wiring of the developing brain (Frost & Schafer, 2016; Konishi et al., 2019; Prinz et al., 2019; Schafer et al., 2012; Schafer & Stevens, 2015; Squarzoni et al., 2014). Microglial trophic support is critical for neuronal survival and the development and homeostasis of oligodendrocytes and their precursors (Ueno et al., 2013; Wlodarczyk et al., 2017). During early postnatal development, microglia are perhaps best appreciated for their role in synaptic pruning (reviewed in Salter & Stevens, 2017), mediated through an experience- and complement-*dependent* mechanism (Schafer et al., 2012; Stevens et al., 2007; Wu et al., 2015). Conversely, a complement-*independent* mechanism has been demonstrated involving the ligand fractalkine and the microglial fractalkine receptor CX3CR1 (Gunner et al., 2019). Knockout of CX3CR1 decreases synaptic pruning and hippocampal–frontal functional connectivity postnatally (Paolicelli et al., 2011; Zhan et al., 2014). When microglial TREM2 receptors were knocked out, mice showed similar deficits in synaptic pruning and connectivity, with alterations predominantly in hippocampus (Filipello et al., 2018). Thus, microglia have critical roles in normal brain developmental processes.

Less is known about the contribution of microglia to adolescent neurodevelopment. As adolescence is a critical period for synaptic pruning, refinement, and myelination—events involving microglia directly—perturbing microglia likely impacts these events (see Figure 1; Schafer & Stevens, 2015). Microglial engulfment of dendritic spines and glutamatergic presynaptic terminals in the PFC is elevated following the peak in spine density in adolescent rats (Mallya et al., 2019), and depletion of microglia during early adolescence (P30) altered glutamatergic spine plasticity via microglia-derived BDNF, impairing learning (Parkhurst et al., 2013). Microglia, through complement-dependent mechanisms, mediated down-regulation of dopamine receptors in the NAc during adolescence, which affected social play behavior in male, but not female, rats (Kopec et al., 2018). This exciting finding aligns with the hypothesis that factors that impact the neuroimmune system could have widespread effects on systems that are being developmentally fine-tuned during this critical period.

In adult animals, microglia are necessary for myelin homeostasis, promoting survival of oligodendrocyte precursor cells and phagocytosing myelin (Hagemeyer et al., 2017; Li & Barres, 2018). However, the precise nature of microglia–oligodendrocyte interactions during the significant myelination that takes place throughout adolescence remain to be elucidated (Giedd et al., 1999). Another developmental process modulated by microglia is adult neurogenesis, levels of which begin to asymptote in adolescence (Ekdahl et al., 2009; He & Crews, 2007; Wooden et al., 2021). In adult animals, microglial effects on adult neurogenesis depend upon their phenotypic state (Ekdahl et al., 2009): Inflammatory microglia reduce adult neurogenesis (Ekdahl et al., 2003; Monje et al., 2003), while noninflammatory microglia are beneficial (Butovsky et al., 2006; Kreisel et al., 2019; Sierra

et al., 2010, 2014). Finally, recent data indicate that microglia have important homeostatic function in regulating neuronal activity, acting as a negative feedback mechanism by detecting and suppressing excess neuronal activation in the healthy brain and following insult (Badimon et al., 2020; Cserep et al., 2020; Pfeiffer & Attwell, 2020).

THE EFFECT OF ALCOHOL ON MICROGLIA IN ADOLESCENT MODELS: A REVIEW OF THE EVIDENCE

It is generally well accepted that alcohol affects the neuroimmune system; however, the precise nature and mechanisms of the effect are debated (summarized in Coleman & Crews, 2018; Erickson et al., 2019; Guerri & Pascual, 2019a,b; Melbourne et al., 2019). The most convincing evidence that the neuroimmune response is relevant for AUDs is that bacterial or viral mimetics escalate alcohol consumption in rodents (Blednov et al., 2011), an effect reversed by the microglial "inhibitor" minocycline (Agrawal et al., 2011), in addition to the observation of an activated microglial morphology in the brains of humans with AUDs (He & Crews, 2008). Activation of the neuroimmune system, including microglia, is apparent in adult animals: Subtle to moderate changes in morphology, up-regulation of cell surface proteins, and changes in cytokine and/or growth factor expression have been observed (reviewed in Melbourne et al., 2019). The nature of immune and microglial alterations, however, varies according to the species, duration and pattern of alcohol administration, and outcome measure (e.g., Bell-Temin et al., 2013; Doremus-Fitzwater et al., 2014, 2015; Gano et al., 2016; Guergues et al., 2020; Marshall et al., 2013; Qin et al., 2008; Rath et al., 2020; Zahr et al., 2010). Furthermore, many of these studies have failed to consider microglial phenotype (Melbourne et al., 2019). Based on the presence of cytokines and DAMPs, several groups hypothesize that proinflammatory microglial activation produces brain volume loss (Crews et al., 2016; Vetreno et al., 2018). However, these immune signals are not specific to microglia nor are they a robust indicator of an inflammatory state. For example, some extent of increase in NF-κB activation or even TNF-α levels are necessary aspects of the immune response, whether that response is pro- or anti-inflammatory (Nennig & Schank, 2017; Scherbel et al., 1999).

Even less is known about the effects of adolescent drinking on the neuroimmune system, specifically microglia. Excessive alcohol use in adolescence is associated with even greater structural changes and damage to the brain than seen in adults (Crews et al., 2000). The loss of brain mass may be due to cell death, altered myelination or synaptic connectivity, and/or impaired neurogenesis (Crews & Nixon, 2009), all of which could involve microglial activation (Crews et al., 2019). Given the sensitivity of the adolescent brain to the damaging effects of alcohol and the potential life-long impact from microglia altering developmental trajectories, this gap is striking. Therefore, to better elucidate converging results, we summarize existing literature for effects of adolescent alcohol exposure on (a) microglia-specific markers and cell morphology, (b) induction of immune molecules related to microglia, and (c) evidence that microglia are primed by alcohol.

Markers and morphology

In adolescent models of alcohol misuse, morphological changes and increased cell surface marker expression signifying microglial activation are consistent, but the activation phenotype is not clear. As detailed in Table 1, increased binding of translocator protein 18 kD ligands using PET imaging was observed after EtOH exposure in adolescent nonhuman primates and rats, indicating microglial activation but not phenotype specificity (Marshall et al., 2013; Saba et al., 2018; Tournier et al., 2020). Increased immunoreactivity or immunopositive (+) cell numbers using Iba1, the calcium binding protein specific to microglia, are observed in regions such as the cortex, hippocampus, amygdala, and substantia nigra in multiple adolescent rat alcohol models (Table 1). In mice, however, Iba1 immunoreactivity is mixed, while microglial numbers are *decreased* (Table 1). These discrepancies may be due to the time point examined. As one rat model shows, microglial number initially declines with 2 days of binge exposure (Marshall et al., 2020) followed by proliferation of microglia in withdrawal, a hallmark of activation (McClain et al., 2011a; Nixon et al., 2008). Further, immunoreactivity could reflect increased protein expression independent of changes in microglial number, which highlights the importance of quantifying cell number. Another hallmark of activation, elevated complement receptor 3 immunoreactivity (CD11b + IR), is observed in frontal cortex and limbic regions after chronic intermittent, 4-day binge, but not acute, EtOH exposure in adolescent rats, persisting into adulthood with chronic intermittent exposure (Table 1).

Notably, in all of these studies microglial processes remained ramified, though thickened compared with controls, consistent with low level activation (Beynon & Walker, 2012; McClain et al., 2011b; Walter et al., 2017). Furthermore, several studies in rodents report microglial loss, which may be more detrimental for synaptic plasticity and development than activation (Grifasi et al., 2019; Hu et al., 2020; Marshall et al., 2020). Only limited studies have reported M1-like markers after alcohol exposure in adolescent rats (Table 1). Flow cytometric analysis of microglia showed increased expression of both M1 and M2 markers after binge exposure (Peng & Nixon, 2021). Some MHCII-positive cells were noted in the dentate gyrus after binge-like exposure and CD68 was detected in hippocampus after chronic alcohol drinking, but both showed ramified morphologies (e.g., see Figure 2 in Li et al., 2019) inconsistent with a proinflammatory phenotype.

Immune molecules

A number of studies in adolescents report EtOH-induced increases in mRNA or tissue protein expression of cytokines, chemokines, and other inflammatory mediators in the PFC and hippocampus (Table 1). While these results support the interpretation that neuroimmune activation is a consequence of alcohol exposure in adolescence, gene expression changes do not necessarily predict changes in protein. In contrast, others have observed a blunted cytokine response to acute EtOH in adolescence versus adults (Table 1). This observation has been interpreted as a relative immaturity of neuroimmune function (Doremus-Fitzwater et al., 2015), but the specific role of microglia is not clear. Indeed, for all of these reports on cytokine effects after alcohol exposure, the source of the cytokine or growth factor proteins is not clear as whole tissue homogenates are used. This point includes the hallmark proinflammatory cytokine TNF-α, the basis of the neuroimmune hypothesis of addiction

(Crews et al., 2011). As detailed in Table 1, more studies report a lack of effect in robust measures of TNF-α, such as protein or microglia-specific TNF-α gene expression.

Similarly, a role for the DAMP, HMGB1, in AUD development is compelling but not specific to microglia nor necessarily proinflammatory. Adults with a history of adolescent-onset AUD have increased HMGB1, an effect not only mimicked in adolescent alcohol models but also observed in comorbid conditions such as stress or depression (Coleman et al., 2018; Franklin et al., 2018; Swartzwelder et al., 2019; Vetreno & Crews, 2012; Vetreno et al., 2013; Weber et al., 2015). HMGB1 is released from dying neurons (Scaffidi et al., 2002) as observed in a rat AUD model (Wang et al., 2015). As a DAMP, it activates neuroimmune signaling and may activate or prime microglia, but outcomes may be pro- or anti-inflammatory. Thus, increased expression of cytokines and other innate immune molecules implicate neuroimmune activation in response to alcohol, but a causal role of these factors in adolescence remains unclear.

Microglial phenotypes and priming

Demonstration of microglial activation is not sufficient to infer that these cells produce cell death and structural damage (Marshall et al., 2013). M2 “anti-inflammatory” microglia also up-regulate cell surface markers, and morphology becomes hyperramified (Beynon & Walker, 2012). Secondary waves of cell death, as would be predicted from M1-like microglia, have not been reported in alcohol models (e.g., Crews et al., 2000; Kelso et al., 2011). Furthermore, the pattern of cell surface and phenotypic markers and gene expression changes in microglia isolated from adolescent rats after alcohol dependence support that microglia are M2-like, consistent with being primed (Peng & Nixon, 2021).

Observation of a heightened microglial response to subsequent challenge—the definition of priming—confirms this hypothesis (Perry & Holmes, 2014). In an adult rat model, microglia show a heightened response upon a double versus single binge, though adolescents have not been reported (Marshall et al., 2016). Chronic alcohol exposure in adolescence resulted in a heightened microglial response (increased CD11b + IR) to restraint stress in adulthood, indicating microglial priming (Walter et al., 2017). Preliminary observations of microglial hyperresponse to LPS stimulation following an alcohol binge (Peng and Nixon, 2018) correspond to reports of increased presence of priming factors such as the DAMP, HMGB1, in rodent models and human AUD (Coleman et al., 2018; Swartzwelder et al., 2019; Vetreno & Crews, 2012; Vetreno et al., 2013). EtOH applied directly to neurons in culture increases release of HMGB1 (Wang et al., 2015), and its expression is increased in human postmortem OFC in subjects with AUD (Crews et al., 2013; Crews & Vetreno, 2014; Flatscher-Bader et al., 2005; Lewohl et al., 2000). Thus, alcohol may prime microglia through neuronal damage producing extracellular DAMPs such as HMGB1.

ARE ADOLESCENTS MORE SUSCEPTIBLE TO MICROGLIA-BASED EFFECTS?

Critical developmental processes occur during adolescence that increase the susceptibility of the brain to the consequences of EtOH (Crews et al., 2000, 2007; White & Swartzwelder,

2004). One consequence is damage, which is distinct in the adolescent brain when compared directly to adults (Crews et al., 2000). The hippocampus is a particular target of alcohol toxicity via effects on cell death and birth (De Bellis et al., 2000; McClain et al., 2014; Morris et al., 2010a; White & Swartzwelder, 2004), both mechanisms of which are modulated by microglia (Ekdahl et al., 2009). Whether microglia are impacted by alcohol differently in adolescents versus adults is not well described. There are few direct comparisons, work from our own laboratories included, though there are a few studies of adolescents or adults by the same investigators with identical approaches that allow for strong comparisons. Furthermore, *valid* comparisons between ages requires comparable BALs. There is some evidence of faster rates of EtOH clearance in adolescent animals, but the presence of significant pharmacokinetic differences varies widely across routes of administration, species, strains, and sex (Walker & Ehlers, 2009; Watson et al., 2020, but see Carrara-Nascimento et al., 2017; Fleming et al., 2019; Lacaille et al., 2015; Morris et al., 2010b; Silveri & Spear, 2000; Vore et al., 2021). Importantly, where EtOH metabolism differences were observed, adolescents typically showed quicker clearance and therefore lower BALs despite greater pharmacodynamic effects. Thus, the slight metabolic differences may not underlie their susceptibility in brain outcomes. Regardless, BALs are critically necessary for rigorous valid comparisons of age differences. In addition, drinking-based models are not good comparisons as adolescent rats consume more alcohol than adults, which produces different BALs and confounds interpretations (e.g., Chung et al., 2008 versus Morris et al., 2010b). Titrated binge models or vapor exposure can overcome slight pharmacokinetic differences between ages as observed in males (Morris et al., 2010b; Varlinskaya & Spear, 2004; Walker & Ehlers, 2009).

For microglia, adults and adolescents seem similar *prima facie*. Two recent papers show similar microglial dystrophy and loss in the hippocampus in adolescents and adults or in adolescents versus aging rats after days of binge-like exposure (Grifasi et al., 2019; Marshall et al., 2020). Another pair of articles described subtle differences in cytokine expression but increased microglial reactivity—more regions and timepoints had increased Iba1 + IR in *adults* (Sanchez-Alavez et al., 2019b) versus adolescents (Sanchez-Alavez et al., 2019a) despite similar BALs during 35 days of EtOH vapor. For microglial phenotype, both ages show mixed phenotypes with a distinct increase in M2-like microglia (Peng et al., 2017; Peng & Nixon, 2021). The consequences of these effects in adolescents, however, may be where adolescents have greater vulnerabilities. Effects may be more severe and persistent by diverting microglia from their homeostatic or developmental roles, impacting brain structure and function long term.

EMERGING EVIDENCE FOR A ROLE OF MICROGLIA IN ADDICTION BEHAVIOR

The implications of microglial activation on adolescent development of addiction behavior remain unclear. In adults, depleting microglia prevented some, but not all, of the neuroimmune responses to alcohol, preventing the escalation of drinking after alcohol dependence, but not reducing voluntary drinking (Walter & Crews, 2017; Warden et al., 2020). These results point to a role of microglia on a symptom of addiction, escalation

of intake, which is similar to what occurs after adolescent exposure. Speculating on how microglia influence increased adult drinking after adolescent exposure requires us to revisit the factors in adolescent development that impact consumption and/or predict future addiction behaviors.

Impairments in PFC-related behavior are well established to increase risk for addiction (Koob & Volkow, 2016), and adolescent alcohol impairs PFC-dependent functions such as impulse control, behavioral flexibility, delay discounting, and working memory (Boutros et al., 2015; Seemiller & Gould, 2020; Sey et al., 2019). Little is known about the role of microglia in these behaviors, and most evidence related to immune signaling is not microglia-specific. Enhanced immune signaling in the PFC after adolescent EtOH exposure was associated with reversal learning deficits and increased perseverative behavior in adult rats tested in the Barnes maze (Vetreno & Crews, 2012). Increased expression of immune markers was seen in the PFC of human postmortem AUD and associated with drinking onset in adolescence, an effect mimicked in animal models after AIE exposure (Crews et al., 2013; Vetreno et al., 2013). Interestingly, adolescent rodents were protected from effects of alcohol, such as neuroimmune activation, cell death, and increased drinking, in $TLR4^{-/-}$ mice or by treatment with the anti-inflammatory indomethacin (Montesinos et al., 2015, 2016; Vetreno & Crews, 2018). However, the role of TLR4 in alcohol drinking was discounted in a comprehensive study in adult mice (Harris et al., 2017) and whether any of these effects involve microglia specifically is not established. Thus, major gaps remain in our understanding of the role of microglia in PFC-controlled behaviors in AUD development.

Alterations in mesolimbic reward circuity that influences reward processing may underlie the adolescent's increased susceptibility for developing alcohol problems in adulthood (Hill et al., 2009; Stice et al., 2013). While there is evidence for differential sensitivity to alcohol-induced neuroadaptations in the mesolimbic system in adolescents versus adults (Alaux-Cantin et al., 2013; Jacobsen et al., 2018; Liu & Crews, 2015; Nees et al., 2015), how microglia influence these changes is difficult to disentangle from the few neuroimmune effects reported. For example, nalmefene, an opioid receptor and TLR4 antagonist approved for reduction in alcohol consumption in patients with AUD, reduced microglial activation and other neuroimmune effects in the striatum and NAc, but astrocytes may be the site of action (Montesinos et al., 2017; Tournier et al., 2020). A role for BDNF has been described in reward processing in adolescents, with specific gene polymorphisms differentially impacting reward processing and striatal function and associated with adolescent drinking onset (Nees et al., 2015). Microglia release BDNF and other growth factors depending on their phenotype, functions that are affected by alcohol exposure in adolescence (Marshall et al., 2020; Peng & Nixon, 2021). Loss of microglia-derived BDNF would have huge implications for striatal function, for example increasing alcohol drinking (Jeanblanc et al., 2009), which is consistent with alcohol preferring rats having lower BDNF levels (Yan et al., 2005).

The hippocampus plays a broad role in behavior (Abela et al., 2013; Rubin et al., 2017), and accordingly, alcohol's neurotoxic effects in hippocampus relate to broad impairments in behavior (De Bellis et al., 2000; Jacobus & Tapert, 2013; Nixon et al., 2011; White

& Swartzwelder, 2004). Examples of the relationship between neuroimmune activation and impairments in hippocampal integrity are numerous in the literature including human (Ward et al., 2009b) and animal models (Ji et al., 2018; Ward et al., 2008), though again not necessarily specific to microglia. The loss of microglia and its role in hippocampal development and function could be more detrimental, but this has not been tested mechanistically (Hu et al., 2020; Marshall et al., 2020). A number of groups report alcohol effects on microglia and adult neurogenesis in adolescence, but mechanistic ties between microglia and neurogenesis specifically are lacking (Ji et al., 2018; Liu & Crews, 2017; Morris et al., 2010a; Vetreno & Crews, 2015; Vetreno et al., 2018; Zou & Crews, 2012). These interactions are actively being pursed in adult and adolescent AUD models (Alvarez Cooper et al., 2020; Nixon et al., 2008).

CONCLUSION

We critically review how adolescent alcohol exposure effects microglia and the implications for AUD pathogenesis. We focused on microglia not only because of their varied roles in homeostatic processes but also because of their dual response to infection and insult, that is, phenotypes, that have been overlooked in the alcohol field (Prinz & Priller, 2017). We predict that microglia have a greater impact on adolescents due not only to relatively subtle effects on addiction relevant behaviors but perhaps more extensive effects on developmental trajectories. However, much remains to be studied. It is difficult to disentangle effects of neuroimmune signaling in general with that of microglia in particular due to a lack of specific tools to manipulate microglia, though these are evolving. Further, few studies make direct, valid comparisons between adult and adolescent animals with BALs controlled. We describe microglial priming, essentially innate immune memory, as a means by which these early insults have long-lasting effects on the brain. There is strong evidence that adolescent alcohol exposure primes microglia persistently. Given the role of microglia in neurodevelopment and in maintaining CNS homeostasis, perturbations of these cells, especially during critical periods of plasticity, have serious implications for brain development, function, and AUD pathogenesis.

ACKNOWLEDGMENT

The authors thank the National Institute on Alcohol Abuse and Alcoholism, the National Institute on Drug Abuse, the National Institute of Neurological Disorders and Stroke for support of R01AA025591 (KN), R21AA025563 (HP/KN), R61NS111081 (JP), T32AA007471 (JKM), and T32DA35200 (CC), and the University of Kentucky College of Pharmacy for funding various works described herein.

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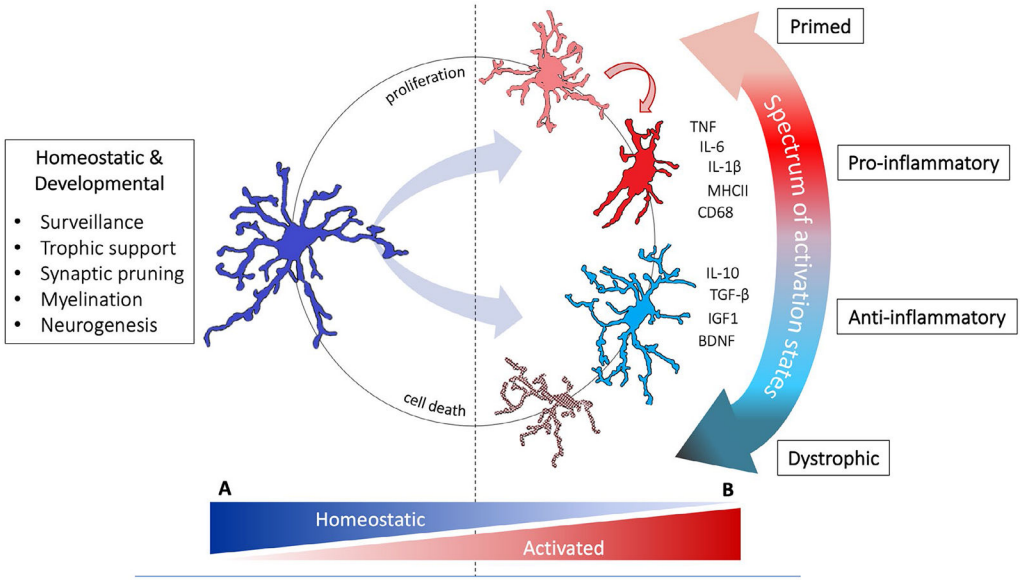

FIGURE 1.

Microglial phenotypes and functions. (A) Healthy adolescent brain. Microglia maintain CNS homeostasis and contribute to ongoing neurodevelopmental fine-tuning by surveying their environment through frequent contacts with synapses and other glia (Nimmerjahn et al., 2005; Tremblay et al., 2010; Wu et al., 2015). This active surveillance accompanied by the microglial sensome, the wide array of receptors these cells use to sample their environment, makes these cells exceptionally sensitive to changes in the CNS milieu (Hickman et al., 2013). Microglia have an even wider range of functions than previously recognized (Hammond et al., 2018); not only do they act as primary responders to CNS injury, microglia are intricately involved in nervous system development and homeostasis (Prinz et al., 2019). (B) Adolescent brain exposed to alcohol. Because microglia can respond to activation by releasing high levels of proinflammatory mediators and reactive oxygen species leading to tissue damage and cell death, they are often the first culprit implicated in neuronal damage. When accompanied by secretion of high levels of proinflammatory cytokines and reactive oxygen species, microglia are frequently termed “classical” or “M1” activated (Kreutzberg, 1996; Ransohoff, 2016). However, activation of these cells can also elicit protective functions that limit tissue damage and facilitate repair. The “alternative” or “M2” state is associated with increased secretion of anti-inflammatory cytokines and growth factors, and may explain why ablating microglia is associated with poorer recovery or outcomes in some conditions (e.g., Lalancette-Hebert et al., 2007; Szalay et al., 2016). With advances in single-cell sequencing, mass cytometry, and microglial manipulation techniques, current designations of microglial phenotypes are an oversimplification (Ransohoff, 2016; Salter & Stevens, 2017). Microglial responses in each disease state have distinct profiles that are not easily classified into classical/M1 or alternative/M2 phenotypes. Instead, specific signaling systems and functions are engaged in a context-dependent manner, with beneficial versus detrimental effects similarly contextually determined. For example, microglial phagocytosis may be detrimental in one context, such as in the over-pruning of synapses in Alzheimer's disease, but beneficial in another, such as in the necessary clearance of cellular debris after injury (Salter & Stevens, 2017). Thus, following EtOH exposure

microglia respond in a context-dependent manner and may adopt a spectrum of phenotypes. These phenotypes reflect the net response to insult and namely degeneration and recovery events, but each of these phenotypes impacts ongoing developmental processes and synaptic plasticity

TABLE 1

Microglia-specific and other neuroimmune measures in models of adolescent alcohol exposure

Study	Model	Exposure	Intox/WD/Abs	Mean BAL	Marker	Result
Microglial markers—histology						
Ward et al. (2009a)	Rat	2 to 3 g/kg/day × 21 day	5 days of abs	–	MHCII	⇧ + IR
McClain et al. (2011b)	Rat	4-day binge	WD 7, 30 days of abs	~305 to 410 mg/dl	Iba1, BrdU, ED1, MHC11	⇧Iba1 + BrdU-Prolif ⇔MHCII ⇔ED1
Walter et al. (2017)	Rat	AIE 5 g/kg × 30 days	Intox	~130 to 170 mg/dl	CD11b	⇧⇔+IR
Vetreno et al. (2017)	Rat	AIE 5 g/kg × 30 days	25 days of abs	~180 mg/dl	Iba1, CD11	⇧ Iba1# ⇧ CD11+IR ⇔ED1 IR
Ji et al. (2018)	Rat	4-day binge; 9 to 11 g/kg/day	Intox	–	Iba1	⇧ #
Sanchez-Alavez et al. (2019)	Rat	CIE vapor × 36 days	Intox/24-h WD/28 days of abs	~170 to 185 mg/dl	Iba1	⇧+IR Intox ⇔↓ + IR
Li et al. (2019)	Rat	10% EtOH; 9.7 g/kg/days × 21 days	Intox	~34 to 105 mg/dl	Iba1, ED1	⇧ Iba1# ED1+ (no stats)
Grifasi et al. (2019)	Mouse	DID	Intox	~70 to 100 mg/dl	Iba1	⇧⇔ + IR ↓Iba1#
Hu et al. (2020)	Mouse	3.5 g/kg – acute AIE 3.5 g/kg/d × 14 days	Intox 21 days of abs	~120 to 150 mg/dl	Iba1, BrdU	⇧Iba1+BrdU-Prolif ↓Iba1#
Marshall and McClain et al. (2020)	Rat	2-day binge (9 to 11 g/kg/day)	Intox	~300 mg/dl	Iba1	↓ Iba1# ⇔MHCII ⇔ED1
Peng and Nixon (2021)	Rat	4-day binge	Intox, WD	~404 mg/dl	CD11b, MHCII, CD32, CD86, CD206, cytokines, growth factors	⇧⇔ ⇧⇔ ↓
Microglial markers-PET						
Saba et al. (2018)	Baboon	0.6 g/kg + 0.1 g/kg/h—acute	Intox 7–12 months of abs	~100 mg/dl	^{18}F-DPA-714 (TSPO ligand)	⇧ ⇧
Tournier et al. (2020)	Rat	AIE 3 g/kg × 14 days	Intox	210–270 mg/dl	^{18}F-DPA-714 (TSPO ligand)	⇧
Other neuroimmune measures						
McClain et al. (2011b)	Rat	4-day binge	WD 7, 30 days of abs	~305 to 410 mg/dl	TNF-α	⇔
Vetreno et al. (2013)	Rat	AIE 5 g/kg × 30 days	Intox 24 days of abs	~135 to 165 mg/dl	HMGB1, TLR4	⇧ + IR ⇧ + IR
Montesinos et al. (2015)	Mouse	AIE 3 g/kg × 14 days	Intox 22 days of abs	~180 mg/dl	NF-κB, HMGB1, cytokines, chemokines, other	⇧
Kane et al. (2014)	Mouse	6g/kg/days × 10 days	Intox	~220 mg/dl	Cytokines, CCL2	⇔
Montesinos et al. (2016)	Mouse	AIE 3 g/kg × 14 days	Intox 22 days of abs	~180 mg/dl	NF-κB, MHCII, CD11b	⇧ ⇧
Pascual et al. (2016)	Mouse	AIE 3 g/kg × 14 days	Intox	~165 to 170 mg/dl	Cytokines, chemokines, TLR4, NF-κB	⇧⇔

Study	Model	Exposure	Intox/WD/Abs	Mean BAL	Marker	Result
Doremus-Fitzwater et al. (2015)	Rat	4 g/kg—acute	Intox	~150 to 370 mg/dl	mRNA: cytokines	⇑⇔↓
Vetreno et al. (2017)	Rat	AIE 5 g/kg × 30 days	25 days of abs	~180 mg/dl	NF-κB	⇑+IR
Vetreno et al. (2018)	Rat	AIE 5 g/kg × 30 days	25 days of abs	~170 to 180 mg/dl	NF-κB mRNA: TLRs, HMGB1, cytokines	⇑+IR ⇑⇔
Vetreno and Crews (2018)	Rat	AIE 5 g/kg × 30 days	25 days of abs	~170 to 180 mg/dl	NF-κB	⇑+IR
Swartzwelder et al. (2019)	Rat	AIE 5 g/kg × 16 days	25 days of abs	–	HMGB1, NF-κB	⇑+IR
Sanchez-Alavez et al. (2019)	Rat	CIE vapor × 36 days	Intox/24-h WD/28 days of abs	~170 to 185 mg/dl	Cytokines, chemokines, other	⇑⇔
Li et al. (2019)	Rat	10% EtOH; 9.7 g/kg/days × 21 days	Intox	~34 to 105 mg/dl	TLR4, NF-κB, cytokines	⇑⇔
Vore et al. (2021)	Rat	AIE 4 g/kg × 20 days	21 to 28 days of abs	–	Cytokines, NF-κB	⇑⇔↓

Abbreviations: +IR, immunoreactivity; Abs, abstinence; AIE, adolescent intermittent EtOH; CIE, chronic intermittent EtOH; DID, drinking in the dark; Intox, intoxication; WD, withdrawal.