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8th Biennial International Drug and Alcohol Research Society (IDARS) Conference 2022

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This Special Issue showcases the research advancements and contributions from attendees of the 8th Biennial International Drug and Alcohol Research Society Conference, held in Nice, France. Bringing together leading researchers from around the world, the conference fosters discussions on the biological and neurological effects of drug and alcohol abuse while driving ongoing efforts to develop effective treatment strategies for substance use disorders. The papers in this eBook represent research presented at the conference or authored by its participants. By capturing these insights, this collection aims to advance scientific understanding and inspire further exploration in the field.

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Editorial: 8th biennial international drug and alcohol research society conference 2022

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KEYWORDS

alcohol intoxication, nicotine, gut microbiome, epigenetics, neuroimmune interaction

Editorial on the Special Issue
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Addiction to drugs and alcohol is an increasing substance use disorder (SUD), and public health problem worldwide, that is characterized by the compulsive use of addictive substances despite detrimental consequences for the individual and society. Globally, SUDs including alcohol use disorders (AUDs) affect more than 150 million people, and in the United States, AUDs alone afflict 29 million individuals, causing more than 140,000 deaths annually [1]. This is further complicated by the opioid crisis, which claims more than 100,000 lives every year [2]. The consequences of the COVID-19 pandemic, lockdown and isolation have resulted in excessive alcohol drinking behaviour, drug addiction, opioid overdose and death along with "Long Hauler" symptoms, comorbidity of neuroCOVID disorders, and now in transition to COVID-19 endemic status exacerbated SUDs. With treatment gaps and challenges on how SUDs are linked to dysbiosis, the implication that the gut-brain axis requires more understanding for comprehensive development of effective medications. Since bridging the SUD treatment gap and discovering new, more effective treatment medications are urgent priorities, there is a need for new research strategies and targets for the treatment of SUDs, as currently available therapies help only few that could benefit [2]. To address unmet needs in SUD treatment new frontiers in AI beyond CHATGPT with large quantitative AI, and combinations with advanced sensing may be useful to create new drugs for treating SUDs.

This Special Issue was put together to highlight the research advances and contributions made by some participants of the 2022, 8th biennial International Drug and Alcohol Research Society (IDARS) Conference in Nice, France. The goal of this Special Issue was to capture and present research data, reviews and discussions on the state of knowledge and the future of drug and alcohol addiction, which continues to be a

global problem. Two research articles provided preclinical data using *in vivo* and *in vitro* techniques to evaluate the effects of alcohol, and four comprehensive review articles covered various molecular, gut microbiome, and neuroimmune effects of drugs and alcohol.

The research article by North et al., *"Alcohol and pregnenolone interaction on cerebral arteries through targeting of vascular smooth muscle Ca^{2+} - and voltage-gated K^+ channels of big conductance,"* investigated the effects of alcohol and pregnenolone (PREG) interaction on cerebral arteries in male and female C57BL/6J mice, to address the consequences in humans who might take pregnenolone supplements while binge drinking. The study showed that PREG at low concentrations synergized with alcohol on middle cerebral artery (MCA) constriction. However, this synergism was lost when both PREG and alcohol were studied at higher doses. Additional *in vitro* electrophysiological data acquisition and measurements of cerebral artery diameter provided evidence that PREG and alcohol converge on a common pathway to evoke cerebral artery constriction. Furthermore inhibition of Ca^{2+} and voltage-gated K^+ large conductance (BK) channels by PREG and alcohol involves disruption of allosteric coupling to Ca^{2+} -driven gating. As PREG regulates several physiological processes, the study highlighted that a combination of PREG and alcohol may affect brain artery function. Of note is that AUDs occur in populations aged 65 and older, who may be at risk for cerebrovascular ischemic conditions.

The research article by Roberts et al., *"Alcohol induced behavioral and immune perturbations are attenuated by activation of CB2 cannabinoid receptors,"* investigated how CB2 cannabinoid receptors (CB2Rs) modulate the behavioral and neuroimmune perturbations using conditional knockout (cKO) mice with selective deletion of CB2Rs from dopamine neurons (DAT-*Cnr2*) and in a separate group of mice from microglia (Cx3Cr1-*Cnr2*). Motor function tests in activity monitors and wheel-running activity, rotarod performance, and alcohol preference tests were used to evaluate behavioral alterations induced by alcohol. An ELISA assay was used to determine the levels of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1α (IL-1α), and interleukin-1β (IL-1β) in the hippocampus of mice. The data showed that cell-type specific deletion of CB2Rs from dopamine neurons and microglia differentially altered the behavioral and alcohol preference tests and revealed that cell-type specific deletion of CB2Rs enhanced alcohol-induced inflammation. Pharmacological modification with the non-specific cannabinoid agonist WIN55212-2, reduced alcohol preference in the cell-type specific CB2R cKO and wild-type mice. The findings suggest that the involvement of CB2Rs in modulating behavioral and immune alterations induced by alcohol may be exploited as a potential therapeutic target in AUDs.

In their review, Crews et al., provided an overview of "Epigenetic regulation of microglia and neurons by proinflammatory signaling following adolescent intermittent ethanol (AIE) exposure and in human AUD." A review of epigenetic mechanisms in response to neuroimmune signaling linked to high mobility group box 1 (HMGB1) plays a key cytokine-like molecule associated with brain proinflammatory signals in alcohol-induced changes. AIE-induced changes in neuroimmune gene expression in neurons, microglia and astrocytes increased adult drinking and preference, increasing anxiety and reward seeking. HMGB1 activates multiple proinflammatory receptors that spread proinflammatory receptors, including Toll-like receptors (TLRs) which mediate proinflammatory gene induction. Epigenetic mechanisms of AIE-induced AUD-like pathology have emerged as mechanisms of alcohol-induced changes in rodent and post-mortem human AUD hippocampus. HMGB1, neuroimmune signaling, epigenetic regulation of forebrain cholinergic neurons along with the hippocampal neurogenic niche provides a linkage between AIE and lifelong signaling associated with pathological behavior and hyperkatifeia that affect the development of AUD. Further studies are needed to develop therapeutic targets through anti-inflammatory and cell transcriptomes.

Next, two reviews also focused their attention on adolescent alcohol use. Hauser et al. presented *"Adolescent alcohol and nicotine exposure alters the adult response to alcohol use."* Basic and clinical human research examining adolescent alcohol consumption and preclinical adolescent and adult alcohol consumption in rodents has revealed that adolescent alcohol and/or nicotine consumption/exposure can promote alcohol consumption during adulthood. The review summarized the knowledge on the effects of voluntary alcohol consumption during adolescence on models of adult alcohol consumption from humans to alcohol-preferring rat lines, including modeling of adolescent alcohol consumption and nicotine data. Mechanisms of the effects of alcohol and nicotine on dopamine and cholinergic systems are potential pharmacological targets and include varenicline, cholinesterase inhibitors, bupropion, lobeline and cytisine, which can reverse or prevent some of the deleterious changes during adulthood following adolescent alcohol consumption/exposure. Further research was suggested to identify and develop additional therapeutic targets for AUDs and co-use/abuse effects.

The review by Getachew et al., *"Adolescent alcohol drinking interaction with the gut microbiome: implications for adult alcohol use disorder"* discussed the growing importance of the bidirectional gut-brain axis as crucial for maintaining overall physiological homeostasis. The authors focused on the influence of adolescent alcohol use on the gut microbiota, as there is a high initiation of alcohol use in early adolescence that increases AUD in adulthood. Dysregulation during adolescent neurodevelopment including neuronal refinement and associated aberrant reward and impulsivity along with environmental and non-neuronal factors are contributing factors to neurodevelopmental impairments and AUD in

adulthood. Furthermore, the roles of the gut microbiome and dysbiosis have been implicated in several peripheral and CNS diseases and AUDs. Mechanisms associated with gut microbiome-microglia interactions, including activation of Toll-like receptor signaling and inflammation-associated molecules suggest that bidirectional crosstalk between the gut and brain may influence fetal alcohol spectrum disorder (FASD). The bidirectional crosstalk between the gut and brain influences symptoms of FASD in individuals after birth in adolescent alcohol drinking and AUD. The gut microbiome, nutrients, and aspects of GPCR signaling are potential therapeutic targets in AUDs.

Vigorito and Chang contributed with *"Alcohol use and the pain system."* They reviewed the mechanisms of nociception, nociceptive pain sensation, and pain perception on the contribution of the pain system to alcohol use, misuse, and dependence. The effects of alcohol at all levels of the pain system, such as neuroimmune interactions, molecular aspects of nociception, spinal, supraspinal, and affective-emotional circuits along with maladaptive homeostasis and allostasis, that contribute to the progression of AUDs were discussed and summarized.

In summary, this Special Issue consisting of two research articles and four review articles, provided pre-clinical research data and comprehensive review articles discussing multiple mechanisms associated with the effects of drugs and alcohol and highlighting challenges and treatment gaps for SUDs. Generative Artificial Intelligence (AI) systems have emerged as promising tools to improve individual health outcomes by streamlining diagnosis and treatment with clinical applications. While generative AI holds potential in the field of substance use disorders, caution is required as the functionalities of AI continue to evolve, as do the challenges of substance use disorders [3]. In addressing treatment gaps, AI beyond CHATGPT may offer useful opportunities to identify more urgent and effective treatment medications for SUDs.

Author contributions

EO wrote the Editorial and approved the submitted version.

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All the participants who submitted their studies to this special research topic "Drug and alcohol research strategies and therapeutic targeting for substance use disorders" are recognized for contributing to the knowledge gaps, challenges and need for new research strategies and targets to address the unmet treatment needs of SUDs.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alcohol and pregnenolone interaction on cerebral arteries through targeting of vascular smooth muscle Ca^{2+}- and voltage-gated K^+ channels of big conductance

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Despite the significant number of people who may be taking pregnenolone supplements while drinking alcohol (ethanol), the widely documented cerebrovascular actions of pregnenolone and ethanol, and the critical dependence of cerebrovascular function on cerebral artery diameter, there are no studies addressing the effect of pregnenolone + ethanol in combination on cerebral artery diameter. We investigated this by evaluating the effect of this combination on middle cerebral artery diameter in male and female C57BL/6J mice, both *in vivo* and *in vitro*. The use of de-endothelialized, *in vitro* pressurized middle cerebral artery segments allowed us to conduct a concentration-response study of constriction induced by pregnenolone ± ethanol, in which drug action could be evaluated independently of circulating and endothelial factors. In both male and female animals, pregnenolone at lower concentrations (≤1 μM) was found to synergize with 50 mM ethanol to cause vasoconstriction. In both sexes, this synergism was lost as one or both vasoconstrictors approached their maximally effective concentrations (75 mM and 10 μM for ethanol and pregnenolone, respectively), whether this was evaluated *in vitro* or *in vivo* using a cranial window. Vasoconstriction by pregnenolone + ethanol was abolished by 1 μM paxilline, indicating BK channel involvement. Moreover, cell-free recordings of BK channel activity in cerebral artery myocyte membranes showed that 10 μM pregnenolone and pregnenolone +50 mM ethanol reduced channel activity to an identical extent, suggesting that these drugs inhibit cerebrovascular BK channels *via* a common mechanism or mechanisms. Indeed, pregnenolone was found to disrupt allosteric coupling to Ca^{2+}-driven gating, as previously reported for ethanol.

KEYWORDS

alcohol intoxication, MaxiK channel, cerebral arteries, neurosteroids, vascular smooth muscle

Introduction

Binge drinking is the most common pattern of excessive alcohol consumption in the US [1–3] and thus constitutes a major public health concern. Binge drinking is a pattern of episodic drinking that results in a blood alcohol concentration (BAC) of >0.08% (i.e., >17.4 mM alcohol), which constitutes legal limit of intoxication to drive motor vehicle in most of the US [4, 5]. Binge drinking occurs at all ages: for example, high school students constitute 14% of all binge drinkers [2], while 33% of college students reportedly binge drink between the ages of 21 and 23 years [6, 7]. Binge drinking in adulthood and in the elderly is associated with an increased incidence of cerebrovascular disease, including both ischemic and hemorrhagic strokes [8–10]. Remarkably, a rapid expansion of alcohol use disorders (AUD) is occurring in the population aged 65 and older [11], a group at particular risk for cerebrovascular ischemic conditions.

In turn, pregnenolone (PREG) is a vasoactive neurosteroid that regulates several physiological processes, including growth and differentiation of glial cells and neuronal firing in the developed brain [12]. PREG supplementation is proposed for the treatment of psychological, mental, and substance use disorders, including AUD [12–18]. There are recent studies suggesting that fluctuations in PREG concentration, as a result of either pathophysiological conditions or therapeutic intervention, could impact not only neuronal but also cerebrovascular function [19, 20]. Therefore, there is potential for an expansion of the segment of the human population that may be engaging in simultaneous intake of PREG and alcohol, a combination that will very likely affect brain artery function.

Ethanol (EtOH) at concentrations reached in the blood during binge drinking constricts cerebral arteries in a wide variety of species, including humans, both *in vivo* and *in vitro* [21–28]. This EtOH action is independent of circulating and endothelial factors; instead, it results from inhibition of the Ca^{2+}- and voltage-gated K^+ large conductance channels (BK channels) present in cerebrovascular smooth muscle (SM) [29]. This EtOH action is consistent with the well-established facts that BK channel activation and inhibition lead to cerebrovascular SM relaxation and contraction, respectively, and thus, cerebral artery dilation and constriction [30–32]. Cerebrovascular SM BK channels include channel-forming α (cbv1 channel isoform; [33]) and regulatory β1 subunits [32, 34]. The latter are necessary both for inhibition of cerebrovascular SM BK channels and for eventual cerebral artery constriction by EtOH [25]. In particular, the β1 transmembrane domain (TM) 2 serves as an EtOH sensor [35].

Our group has recently documented the fact that PREG, at local and therapeutically relevant concentrations (sub-to low μM), also inhibits cerebrovascular SM BK channels, eventually inducing constriction of cerebral arteries [19]. In contrast to EtOH, these PREG actions do not require β1 subunits; instead, cbv1 channels suffice [19]. While the separate effects of alcohol and PREG on SM BK channels and cerebral artery diameter have been investigated, the effect of concomitant administration of PREG + EtOH on SM BK activity and cerebral function has not been addressed, despite its important epidemiological and public health implications. To address this issue, we here evaluate the effect of *in vivo* and *in vitro* EtOH+/-PREG administration to the middle cerebral artery (MCA), which provides most of the blood flow to the brain and is most commonly affected by neurovascular ischemia and AUD [36–39]. To obtain mechanistic insights, we evaluate EtOH+/-PREG actions on MCA SM BK channel activity in cell-free systems. Our study reveals that PREG at submaximal constrictive concentrations synergizes with EtOH, thus amplifying the MCA constriction induced by EtOH concentrations (50 mM) obtained in the blood during binge drinking. This synergism is lost when both agents are probed at or close to their maximally effective concentrations, which is explained by their shared targeting of allosteric mechanisms that result in disruption of Ca^{2+}-driven channel gating.

Materials and methods

Ethical aspects of the research

The animal care and experimental protocols were reviewed and approved by the IACUC of the University of Tennessee Health Science Center, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

In vivo measurements of cerebral artery diameter

C57BL/6J mice of both sexes, all 8–12 weeks old, were anesthetized with a mixture of xylazine/ketamine (12/100 mg/kg of weight) and kept anesthetized for the duration of the experiment with subsequent ketamine doses (50 mg/kg of weight) every 15 min or as needed. A catheter was inserted into the internal carotid artery so that experimental drug infusions were directed toward the brain rather than the thoracic cavity. An area of the skull was cleared of tissue and thinned in order to visualize the branching arteries originating from the middle cerebral artery (MCA) on the brain side where the catheter was inserted, above the zygomatic arch, between the ear and eye [27, 40]. The arteries branching out from the MCA were monitored using a Leica MC170 HD microscope with a mounted camera (Leica M125 C) connected to a computer monitor. Drugs were diluted to their final concentration in 0.9% NaCl and administered via catheter at 0.1 mL/25 g of mouse weight. Cranial window images before and after drug administration

were acquired every 60 s for later analysis; a sample of n = 5–6 was acquired for each group (with n representing the number of separate animals).

In vitro measurements of cerebral artery diameter

Male and female C57BL/6J mice, all 8–12 weeks old, were deeply anesthetized with isoflurane *via* inhalation using an open-drop method in a bell jar. Upon losing their response to toe pinch, animals were quickly decapitated with sharp scissors. Resistance-size MCAs (~100 μm in outer diameter) were dissected from the mouse brains. Endothelium was removed by passing an air bubble through the vessel lumen for 90 s [29]. Arterial segments (0.5 cm long) were cannulated at each end, and the artery exterior was continuously perfused with physiologic sodium saline (PSS) of the following composition (mM): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 0.023 EDTA, 11 glucose, and 24 $NaHCO_3$; pH = 7.4, at 35°C–37°C. PSS was continuously bubbled with $O_2/CO_2/N_2$ at 21/5/74%. Vehicle control (dimethyl sulfoxide; DMSO), PREG, EtOH, or the PREG + EtOH combination were diluted into PSS and perfused over the arterial segment. The artery external wall diameter was measured using the automatic edge-detection function of the IonWizard software package (IonOptix) via a Leica MC170 HD microscope with a mounted camera (Leica M125 C) connected to a computer monitor.

Electrophysiology data acquisition and analysis

For all electrophysiological recordings, whether in mouse cerebral artery myocytes or following heterologous expression of recombinant BK channels in *Xenopus laevis* oocytes, ionic currents were recorded from excised membrane patches in the inside-out (I/O) patch-clamp configuration. Patch-recording electrodes were pulled from glass capillaries and treated as described previously [41]. When filled with high K+ solution (see below for composition of electrode solutions), the vast majority of tip resistances were ~2 MΩ, with a few reaching 5 MΩ. Series resistance was electronically compensated up to 80% by the EPC8 amplifier. In all experiments, whether on myocytes or oocytes, the nominal free $[Ca^{2+}]$ in experimental solutions was calculated with MaxChelator Sliders (Stanford University) and validated experimentally using Ca^{2+}-selective and reference electrodes [42]. Solutions were applied to the cytosolic side of the patch using an automated, pressurized Octaflow system (ALA Scientific) through a micropipette tip with an internal diameter of 100 μm. Experiments were carried out at room temperature (20°C–22°C). Ionic currents at single-channel resolution were recorded using an EPC8 amplifier (HEKA) at 1 kHz. Data were digitized at 5 kHz using a Digidata 1320A A/D converter and pCLAMP 8.0 (Molecular Devices).

For ionic current recordings from MCA smooth muscle BK channels, cerebral artery myocytes were isolated from adult mouse MCA as described in detail elsewhere [43]. Bath and electrode solutions contained (mM): 130 KCl, 5 EGTA, 1.6 HEDTA, 2.28 $MgCl_2$ ($[Mg^{2+}]_{free}$ = 1 mM), 15 HEPES; pH 7.4. Free $[Ca^{2+}]$ in the solution (30 μM) was adjusted to the desired value by adding $CaCl_2$. An agar bridge with Cl^- as the main anion was used as a ground electrode.

For ionic current recordings in *Xenopus laevis* oocytes, isolated oocytes (stages V and VI) were purchased from *Xenopus* 1. Oocytes were defolliculated with forceps under a microscope and stored at 18°C until injection with cbv1-coding cRNA injection. Each oocyte was injected with 23 nL of 40 ng/μL cbv1 cRNA, with patch-clamp recordings being conducted 36–72 h after injection. Immediately before patch recordings, each oocyte was manually freed from its vitelline layer as described [41]. Both bath and electrode solutions contained (mM) 135 K^+ gluconate, 5 EGTA, 2.28 $MgCl_2$, 15 HEPES, and 1.6 HEDTA, pH 7.4. As for solutions used with myocyte experiments, free $[Ca^{2+}]$ in the solution (30 μM) was adjusted to the desired value by adding $CaCl_2$. An agar bridge with K^+ gluconate as the main anion was used as a ground electrode [41]. Two major Ca^{2+}-dependent gating parameters, i.e., the Ca^{2+} dissociation constant (K_d) and the allosteric factor (C) that couples Ca^{2+}-binding to channel close-open transitions in absence of stimuli, were estimated from the Ca^{2+} dependence of Po at very negative voltages; such estimates have been used previously to study the effect of EtOH on the Ca^{2+} gating behavior of cbv1 channels [44]. To do this, we obtained R0, i.e., the NPo ratio in the presence of Ca^{2+} (0.1–100 μM) over the NPo ratio in absence of Ca^{2+} (determined +30 mV) in the absence and presence of 10 μM PREG; data were then fitted using the following equation:

$$R_0\left([Ca^{2+}]\right) = \frac{NP_0[V,[Ca^{2+}]]}{NP_0[V,0]} = \left[\frac{1+KC}{1+K}\right]^4 = \left[\frac{1+C[Ca^{2+}]/Kd}{1+[Ca^{2+}]/Kd}\right]^4$$

Chemicals

Pregnenolone was purchased from Abcam. Ethanol (200 proof; E7023) and all other chemicals were purchased from Sigma Aldrich. PREG stock solution was prepared in DMSO and diluted into saline, PSS, or patch-clamp bath solution immediately before application to the animal, artery, or membrane patch, respectively. Each animal or pressurized artery was exposed to vehicle, PREG, EtOH, or the PREG + EtOH

combination only once in order to avoid any possible receptor (i.e., BK channel) desensitization [45]. Membrane patches were perfused with increasing concentrations of Ca^{2+}, first in the absence and then in the presence of PREG.

Data analysis

Analysis was performed by investigators who were blind to experimental group identity. Changes in artery diameter obtained from cranial window experiments were determined using the ImageJ software package (ImageJ 1.52a, downloaded from https://imagej.nih.gov/ij/download.html). Changes in artery diameter *in vitro* were determined using the IonWizard software package (IonOptix). The product of the number of channels in the membrane patch (N) and the individual open probability (Po) was used as an index of channel steady-state activity. NPo was obtained using a built-in function in pCLAMP 8.0 (Molecular Devices).

Statistical analysis was performed using the InStat3.05 software package (GraphPad). Data distributions were checked using a Kolmogorov–Smirnov approach in cases where the number of observations ≥10. For normally distributed data (Gaussian type), the *t*-test was used to test for statistically significant differences between two groups. For data following a non-Gaussian distribution or whose mode of distribution could not be established with certainty (number of observations <10), the statistical methods employed included the Mann–Whitney test for comparisons between two experimental groups, and the Kruskal–Wallis test followed by Dunn's post-test for comparisons of three or more experimental groups. The threshold for significance was set at $p < 0.05$; group sizes were determined to achieve greater than or equal to 80% power at this significance threshold. Data are reported in the form mean ± SEM. Final data plotting and fitting processes were conducted using the Origin 2020 software package (OriginLab).

Results

Pregnenolone and ethanol administered *in vivo* evoke similar constriction of cerebral arteries without displaying synergism

In order to evaluate the combinatory actions of PREG + EtOH on male and female cerebral arteries at the organismal level, we used the cranial window technique. This technique allows for the continuous monitoring of resistance-size pial arteries that branch out of the MCA, and has previously been used to evaluate the pharmacological effects of each drug on these vessels [19, 27]. Intra-carotid infusion of either volume control (0.9% NaCl) or vehicle control (DMSO) failed to evoke significant changes in MCA diameter when compared to averaged pre-infusion values (i.e., the baseline in Figure 1), which were obtained via continuous artery diameter monitoring for no less than 3 min. For the testing of PREG, a concentration of 10 μM was chosen because this constitutes the lowest PREG concentration that is able to evoke maximal constriction of the mouse MCA *in vitro* and *in vivo* (EC_{max}) [19]. For EtOH, 50 mM was chosen because this concentration is reached in blood circulation after a moderate-to-heavy alcohol consumption episode and is close to EC_{max} for ethanol for constriction of mouse cerebral arteries that branch out of Willis' circle, including the MCA [28]. In contrast to the controls, bolus injections of either PREG or EtOH administered to male animals resulted in 28% ± 3.8% and 31.2% ± 2.1% reductions in artery diameter, respectively (Figure 1). For both agents, maximal constriction was detected around 3 min after bolus injection. The effect of each agent differed significantly from the time-matched effects of either saline or DMSO ($p = 0.0079–0.0043$). In female animals, PREG and EtOH also evoked a peak constriction around 3 min after bolus injection, with arterial diameter decreasing by 21.3% ± 2.2% and 19.5% ± 4.1%, respectively. These vasoconstrictive responses did not differ statistically from those observed in males (Figures 1A, C, E vs. Figures 1B, D, F).

In males and females, concomitant application of PREG + EtOH caused MCA constriction of magnitude 33.6% ± 2.8% and 19.3% ± 3.2%, respectively. Within each sex, the response to the combination PREG + EtOH did not differ statistically from the responses evoked by the individual agents (Figures 1C–F). Since the two agents were applied locally in bolus with the injectate directed toward their site of action (the MCA pial artery branch under recording), the lack of synergism between the PREG and EtOH vasoconstrictive actions is unlikely to have been due to modification of the pharmacokinetic properties (i.e., absorption, distribution, metabolism, and/or elimination) of one drug caused by the simultaneous presence of the other. Rather, the lack of synergism can be explained by (i) the system reaching its maximal level of constriction under each agent and under their combination (a "ceiling effect"), or (ii) convergence of the constrictions elicited by either PREG or EtOH on a given organ/tissue pathway.

Pregnenolone and ethanol converge on a common pathway to evoke cerebral artery constriction

To investigate the possibility that EtOH and PREG constrict MCA through a common pathway, we used a wide range of PREG concentrations (10 nM–100 μM [19]) in the presence and absence of 50 mM EtOH. If the two cerebrovascular constrictors were acting through a common pathway, then submaximal concentrations of PREG in combination with EtOH at 50 mM would show additivity in constricting the MCA [46]. Since we have previously documented that MCA constriction by either

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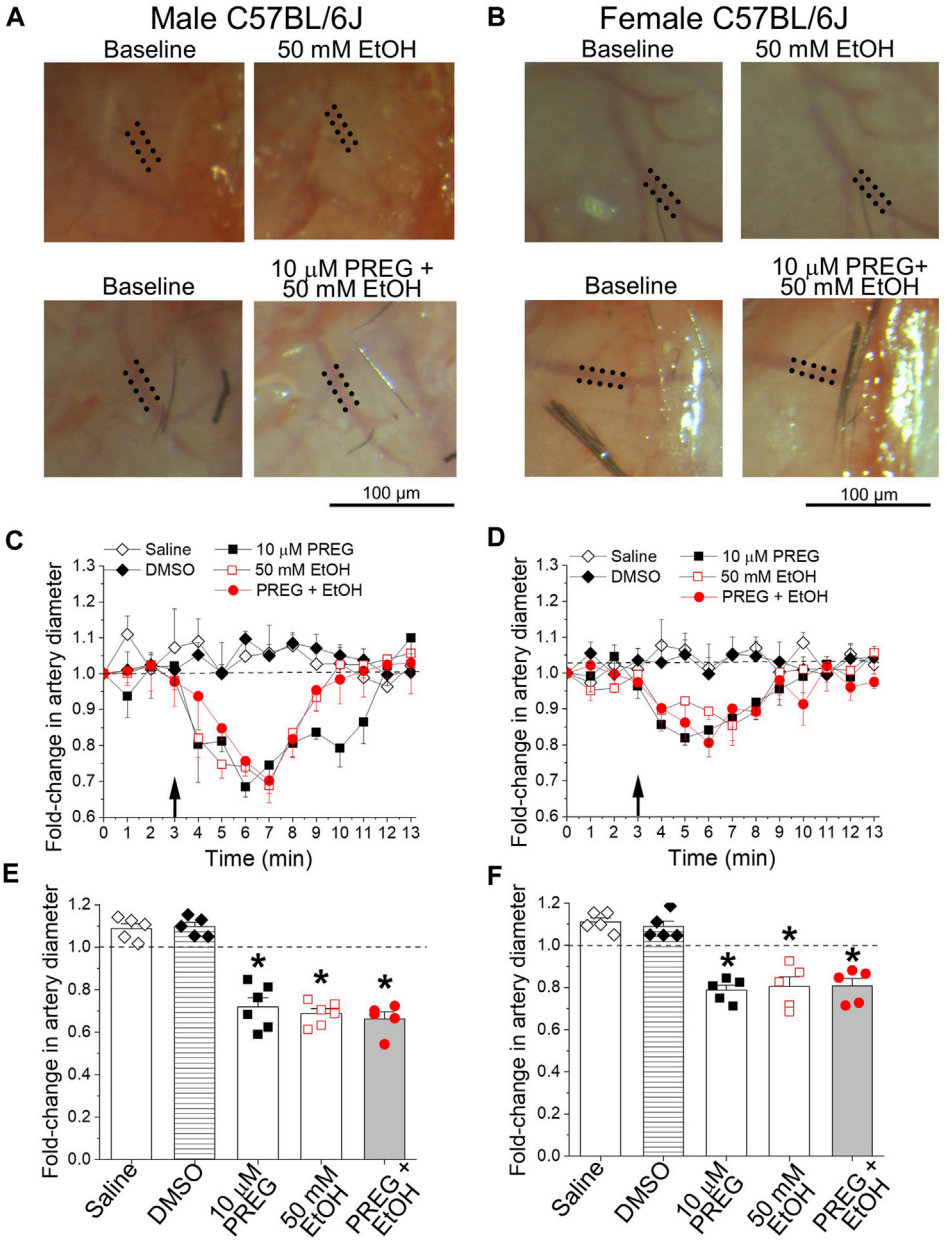

FIGURE 1
At concentrations known to constrict middle cerebral arteries *in vitro*, pregnenolone and ethanol induced constriction of these arteries *in vivo*, the effects of each agent and their combination being of similar magnitude. **(A)** Representative images showing diameter measurement of pial arteries that branch out of the middle cerebral artery (MCA). Images were obtained via a cranial window on male C57BL/6J mice at baseline (pre-drug), and after drug infusion (at 7 min of observation): 50 mM EtOH (top) and the combination of 10 μM PREG with 50 mM EtOH (bottom). Dotted lines highlight outer MCA walls. **(B)** Similar images to those depicted in **(A)**, from age-matched female animals. **(C)** Graph depicting time-dependent changes in pial artery diameter during cranial window recordings for male C57BL/6J mice. The black arrow at minute 3 indicates time of infusion. Here and in **(D–F)**, the horizontal dashed line at 1.0 highlights a lack of effect. **(D)** Graph similar to that shown in **(C)**, showing data from age-matched female animals. **(E)** Average maximal changes in artery diameter from pre-infusion levels compared to volume-matched (saline) and vehicle (DMSO) controls for male mice. **(F)** Graph similar to that shown in **(E)**, showing data from female animals. In both male and female groups: n = 5–6/group, where n represents the number of individual mice; *p-value <0.05.

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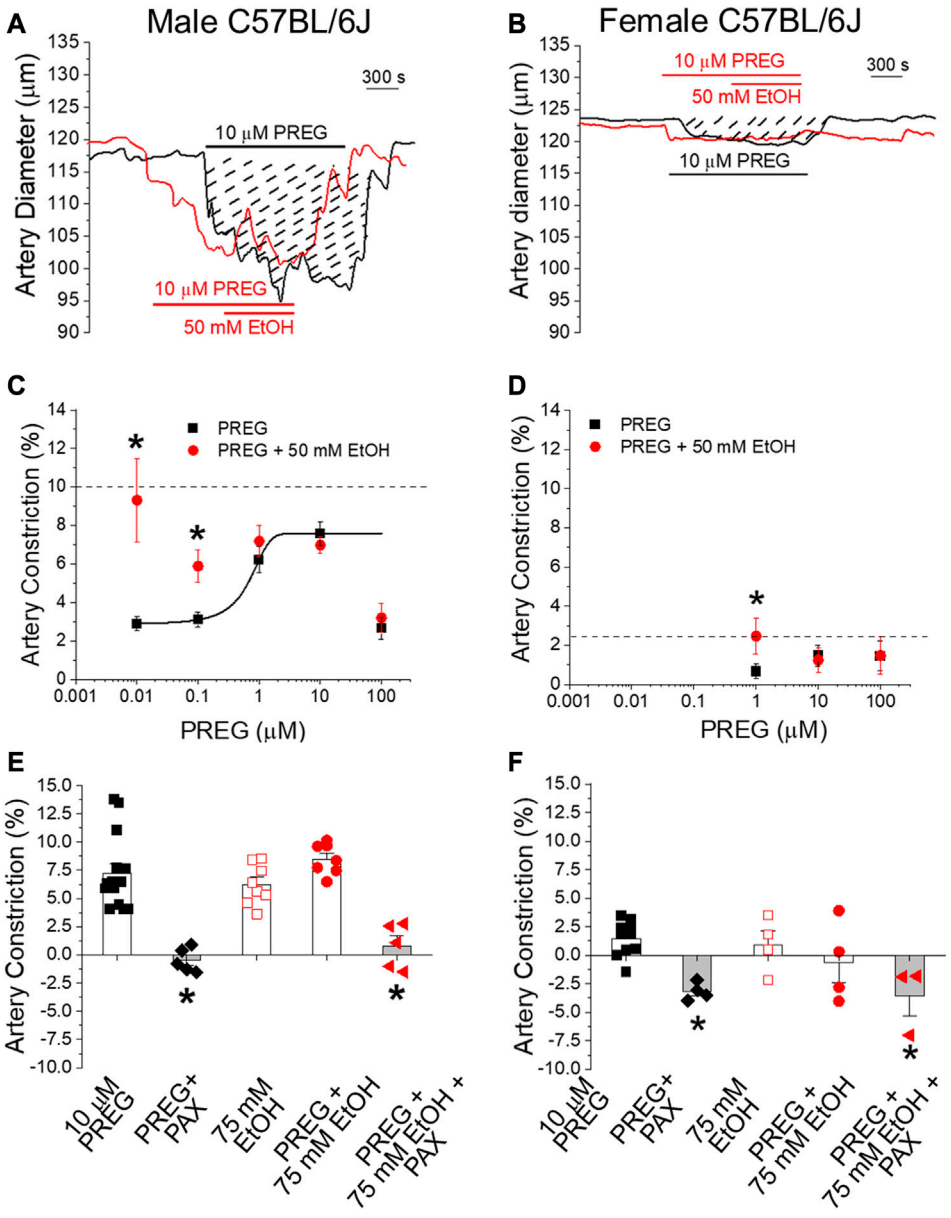

FIGURE 2
The concentration–response curve for pregnenolone-induced *in vitro* constriction of cerebral arteries in the absence vs. presence of ethanol reveals synergism in the vasoconstrictive effect of these agents at lower pregnenolone concentrations. Matching *in vivo* data, synergism is lost when these ligands reach their maximally effective concentrations. **(A)** Representative traces of time-dependent changes in middle cerebral artery (MCA) diameter for male C57BL/6J mice. MCA were de-endothelialized and *in vitro* pressurized at 60 mmHg. The black trace shows MCA constriction by 10 μM PREG. Dashed lines highlight the area under the curve, which is indicative of constriction magnitude. The red trace depicts a similar degree of constriction by 10 μM PREG followed by addition of 50 mM EtOH. **(B)** Representative traces of *in vitro* MCA diameter in female animals following manipulations identical to those described for male animals. **(C)** Averaged change in MCA diameter induced by PREG in males. Datapoints for PREG are in black; datapoints for PREG + EtOH are in red. The horizontal dashed line indicates the average constriction evoked by 50 mM EtOH alone. Concentration-dependent constriction by PREG is fitted to a Boltzmann curve; n = 6–7 for each PREG concentration. **(D)** Average change in MCA diameter induced by PREG in females, with similar details as provided in the description of the data shown in **(C)**; n = 5–7 for each PREG concentration. **(E)** Scatter graphs and average change in MCA diameter in males upon perfusion with the various drugs under investigation and their combinations. **(F)** Average change in MCA diameter in females, with similar details as provided in the description of the data shown in **(E)**. In both **(E,F)**: PAX = 1 μM paxilline; *$p < 0.05$.

agent does not require the endothelium, but is mediated by targets and mechanisms located in the vascular SM [19, 29], MCA segments were de-endothelialized before pressurization, as described in the *Materials and Methods* section.

Remarkably, submaximal concentrations (<EC_{max}, 0.001–0.1 μM) of PREG that evoke constriction displayed synergism with 50 mM EtOH (Figures 2C, D); in males, the degrees of constriction induced by 10 nM and 100 nM PREG in the presence of co-administrated EtOH (9.32% ± 2.16% and 5.88% ± 0.83% constriction, respectively) were significantly greater than the degrees of constriction produced solely by PREG (2.96% ± 0.38% and 3.31% ± 0.38%); $p = 0.0159$ and $p = 0.026$, respectively. Importantly, this synergism was lost at maximally effective and supramaximal concentrations of PREG (i.e., 1, 10, and 100 μM [19]) (Figure 2C), which is to be expected in a case of two agents acting *via* a common pathway or common target(s).

In females, the concentration–response curve of MCA constriction in response to PREG was restricted to evaluate higher concentrations, shown to be effective in our previous publication [19]. Records from these animals also showed synergism between PREG and EtOH when PREG was probed at submaximal concentrations (1 μM): 2.46% ± 0.91% constriction vs. 0.66% ± 0.38% constriction with PREG alone ($p = 0.04206$; Figure 2D). As found with males, MCA constriction in females under exposure to EtOH and PREG was characterized by loss of synergism when PREG was probed at maximally effective concentrations (10 and 100 μM; [19]) (Figure 2D). Since it has been documented by us and others, both in this system and under identical conditions, that depolarizing 60 mM KCl constricts MCA by >20% in both males and females (see [28] and references cited therein), the lack of synergism between PREG and EtOH in evoking MCA constriction cannot be explained by a "ceiling effect" (i.e., by MCA segments reaching their maximal possible degree of constriction). Therefore, the synergism between EtOH and PREG at submaximal concentrations and the loss of synergism when either EC_{Max} is reached on isolated, de-endothelialized MCA segments indicate that the two drugs converge on a common pathway or target(s), likely located in the vascular SM itself.

Given the involvement of BK channels in EtOH- [25, 28, 29] and PREG-induced [19] constriction of de-endothelialized cerebral arteries, we next probed whether these channels were involved in MCA constriction by PREG or EtOH when these agents were applied in combination vs. separately. Synergism in MCA constriction was not detected either in males or in females when EtOH was probed at 75 mM (Figures 2E, F), extending our findings shown in Figures 2C, D. More importantly, in both males and females, paxilline at a concentration that selectively blocks BK channels (1 μM; [47]) completely abolished the constriction evoked by PREG alone and by PREG + EtOH (Figures 2E, F). This outcome indicates that the common pathway implicated in constriction induced by PREG and EtOH involves SM BK channels.

BK channel inhibition by pregnenolone and ethanol involves Ca^{2+}-driven gating

To determine whether SM BK channels were indeed shared targets of EtOH and PREG, we set out to explore whether the lack of synergism in concomitant application of EtOH + PREG could be observed at the level of BK channel activity itself, independently of cell signaling and internal organelles. Thus, we recorded BK channel steady-state activity (NPo) in excised, I/O membrane patches from myocytes freshly isolated from mouse MCA (Figure 3A). We chose 10 μM PREG because this concentration constitutes EC_{max} for PREG-induced constriction of MCA (Figure 2) and for BK channel inhibition by this neurosteroid [19]. The data showed that the inhibition of channel activity by PREG was indistinguishable from that evoked by PREG + EtOH. Indeed, the application of 10 μM PREG to the patch decreased BK NPo to 0.7 ± 0.04 of pre-drug levels (baseline in Figures 3B, C), while the concomitant application of PREG + EtOH decreased NPo to 0.71 ± 0.04 from the baseline (Figures 3B, C). Moreover, the inhibition of BK channel steady-state activity by either 10 μM PREG or 10 μM PREG+50 mM EtOH reported here is identical to the inhibition evoked by 50 mM EtOH alone [25, 29]. Since cerebrovascular SM BK channel inhibition is a well-known mechanism leading to cerebral artery constriction [30, 32], the lack of synergism between EtOH and PREG actions when probed at maximal concentrations in terms of their impact on SM BK channel activity, de-endothelialized MCA segments, and MCA *in vivo* supports the idea that inhibition of cerebrovascular SM BK channels is the common mechanism underlying MCA constriction induced by these drugs (see Discussion).

We have previously documented the finding that EtOH inhibition of BK channels at physiological levels of Ca^{2+} found in cerebrovascular myocytes requires the presence of modulatory, smooth muscle-abundant BK β1 subunits [25]. Moreover, the β1 subunit TM2 acts as a specific EtOH sensor [35]. In contrast, PREG-induced inhibition of these channels does not involve β1 regulatory proteins; instead, channel-forming α subunits suffice for steroid action [19]. While each ligand inhibits BK channel activity through recognition by different subunits that form part of the SM BK channel heteromer, the action of the two ligands must converge on a gating mechanism or mechanisms in order to explain their lack of synergism at maximal concentrations (shown in Figure 3). Therefore, we probed the effect of PREG on BK channel currents mediated by recombinant BK channel proteins cloned from cerebrovascular smooth muscle (cbv1 isoform; *Material and Methods*) and expressed in *Xenopus laevis* oocytes; this system allows for proper comparison with data previously obtained with EtOH under identical recording conditions [44]. Importantly, PREG has been shown to be ineffective in the absence of activating concentrations of Ca^{2+} [48]. Therefore, we focused

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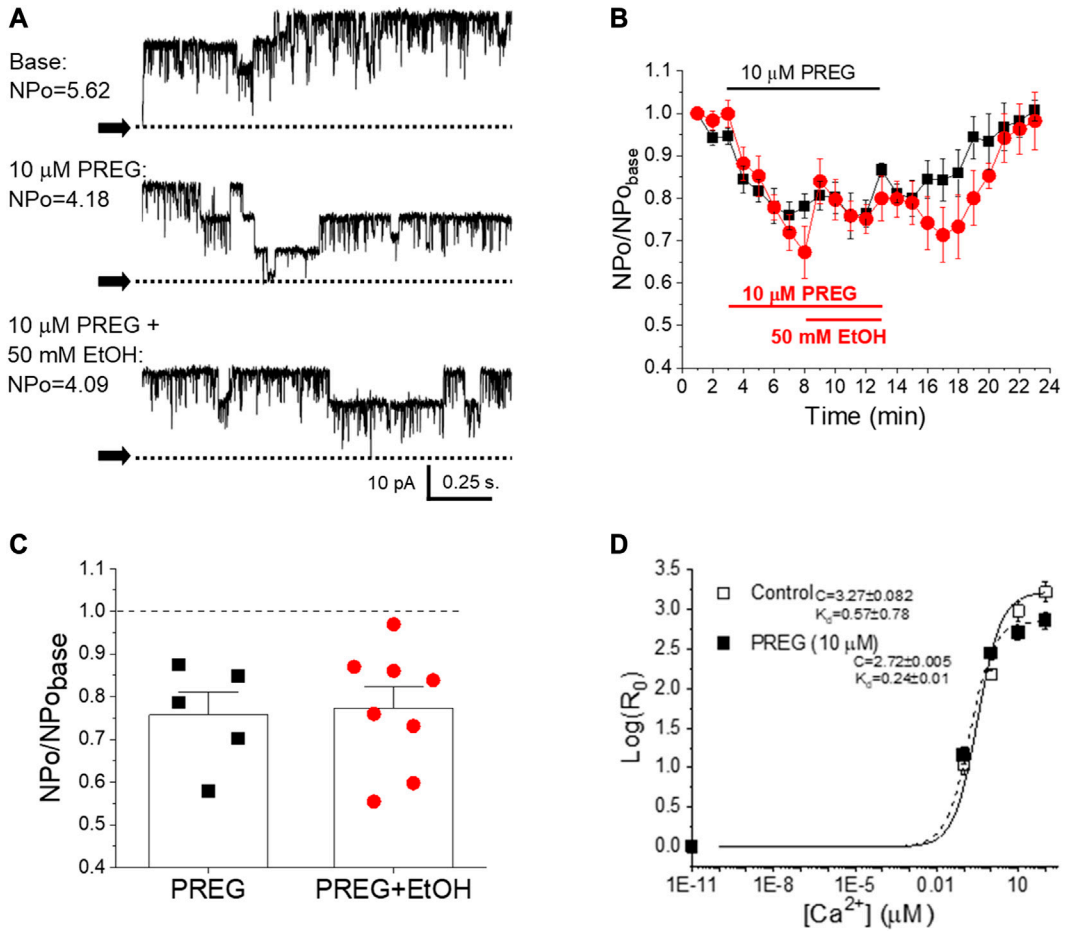

FIGURE 3
There is no synergism in pregnenolone and ethanol inhibition of BK channels as studied in cell-free systems; both ligands disrupt allosteric coupling to Ca^{2+}_i-driven gating. **(A)** Representative records of BK channel activity (NPo) in inside-out patches from freshly isolated MCA myocytes, obtained before bath patch perfusion with control (top), 10 μM PREG- (middle) and 10 μM PREG+50 mM EtOH-containing bath solutions (bottom); $[Ca^{2+}]_{free}$ = 30 μM. N = number of channels in the patch; Po = single channel open probability. **(B)** Averaged, time-dependent BK channel inhibition by 10 μM PREG (black) and 10 μM PREG+50 mM EtOH (red). **(C)** Average changes in single datapoints displaying changes in BK NPo induced by PREG vs. PREG + EtOH. A dashed line indicates lack of drug effect. **(D)** Averaged log [R0]-$[Ca^{2+}]_i$ plots from BK channel-forming cbv1 proteins expressed in *Xenopus laevis* oocytes in the absence and presence of 10 μM PREG, fitted as described in the *Material and methods*. Best-fit parameters (±95% confidence interval) are shown to the left of the plots. R: NPo ratio in the presence (0.01–100 μM) and absence of Ca^{2+}_i obtained at 30 mV; K_d: Ca^{2+} dissociation constant; C: allosteric parameter coupling Ca^{2+}-binding to open-to-closed channel transitions in the absence of Ca^{2+} or voltage-sensor activation; *n* = 3.

on determining the action of PREG on the Ca^{2+}-driven gating of cbv1 channels. Specifically, we derived the changes in the channel's Ca^{2+} dissociation constant (K_d) and the allosteric coupling parameter (i.e., parameter C in the HA model [49]) that links Ca^{2+}-binding to the intrinsic channel gating (i.e., closed-to-open transitions) occurring in the absence of Ca^{2+} binding and membrane depolarization. Both parameters were obtained as described in the *Materials and methods* section. Figure 3D shows that 10 μM PREG, surprisingly, did not increase K_d, but rather decreased it. However, PREG did decrease C, an allosteric decoupling that likely contributes to the inhibitory action of PREG on this channel. Remarkably, these two parameters of cbv1 channel gating are also targeted by EtOH, and the overall effect of this drug on cbv1 channel activity is largely determined by its actions on K_d and C [44]. Whether PREG actions on K_d and C are the primary determinants of overall PREG-induced inhibition of BK channels remains to be confirmed (see *Discussion*).

Discussion

Our study provides both translational and mechanistic information on the cerebrovascular effects of two easily

accessible drugs: PREG and EtOH. PREG-containing formulations (at 500 mg/day) are proposed as therapeutics against prevalent psychiatric and substance-use disorders, including alcohol misuse [12–19]. In turn, moderate-to-heavy episodic alcohol consumption, e.g., "binge drinking," which results in BAC around 50 mM EtOH (as used in the current study), constitutes the most prevalent form of alcohol misuse in the US and other developed countries [1–3]. Moreover, approximately 90% of individuals affected by alcohol misuse disorders will relapse within 4 years, according to the National Institute on Alcohol Abuse and Alcoholism [50]. Therefore, mood-stabilizing supplements containing PREG could be frequently consumed by individuals who binge-drink alcohol. Furthermore, the contribution of cerebrovascular ischemia to prevalent disorders is being increasingly recognized. Indeed, alterations in normal control of cerebral artery diameter play a significant role in the pathophysiology of vascular dementia, migraines, seizures, and cerebral vasospasm [51–53]. While (i) the constriction of cerebral arteries by toxicologically relevant concentrations of EtOH has been widely reported in several species, including humans ([28] and references therein), and (ii) the constriction of cerebral arteries by therapeutically relevant concentrations of PREG has been previously reported by our group [19], the current study is the first to determine the effect of PREG combined with EtOH on cerebral artery diameter. The data clearly demonstrate that submaximal vasoconstrictive concentrations of PREG (subμM), i.e., concentrations equivalent to those found in the blood in humans following administration of PREG supplements, are able to potentiate the constriction of cerebral arteries (MCAs) by 50 mM EtOH (Figure 2). Thus, it is reasonable to propose that the ischemic effects of intoxicating levels of alcohol (≤50 mM) will be potentiated by the presence of PREG (sub-to low μM) in brain circulation, and *vice versa*. Regarding the changes in diameter reported here in response to separate or combined administration of EtOH and PREG (i.e., less than 10% decrease from pre-drug administration values), it is important to underscore that even mild changes in cerebral artery diameter are expected to result in robust alterations in brain perfusion, since according to Poiseuille's law, flow rate is directly proportional to the 4th power of vessel radius [54].

Our study had also documented the finding that, as PREG concentrations and their associated constriction of MCA increased, the synergism with EtOH diminished (Figure 2). Indeed, at concentrations for each ligand that were close to the EC_{max} to evoke MCA (EtOH≥50 mM and PREG≥10 μM), the vasoconstrictive effect of EtOH, PREG, or their combination was similar, whether this was studied *in vivo* through a cranial window (Figure 1) or *in vitro* through isolated MCA segments that had been previously de-endothelialized and pressurized to obtain physiological smooth muscle tone before drug application (Figure 2). These findings are consistent with the involvement of a common mechanism or target in EtOH- and PREG-induced constriction of MCA. The observations that selective channel block by paxilline abolished PREG and EtOH action (Figure 2), and that PREG and EtOH did not show synergism in their inhibitory action on smooth muscle BK channels when studied in free-cell systems (Figure 3), strongly suggest that smooth muscle BK channels themselves are the common effectors of PREG- and EtOH-induced constriction of MCA.

In light of previous findings, our data also constitute important findings from a mechanistic standpoint. On the one hand, it has previously been shown by our group that smooth muscle BK channel inhibition and eventual MCA constriction are dependent on the presence of BK regulatory subunits of β1 type, which are abundant in cerebrovascular smooth muscle [25, 32]. In particular, the TM2 domain of this accessory subunit serves as an alcohol sensor [35]. In contrast, PREG inhibits MCA smooth muscle BK channels and thus evokes constriction via its recognition by the channel-forming subunit [19]. Even though EtOH and PREG are directly sensed by different proteins that participate in cerebrovascular SM BK channel heteromers, the lack of inhibitory synergism in their impact on channel activity at maximal concentrations of these drugs (Figure 3) indicates that both modulators must converge on some gating process(es) to inhibit BK channels. Several pieces of evidence support the idea that EtOH and PREG both modulate Ca^{2+}-driven gating to inhibit cerebrovascular BK channel activity. First, neither EtOH [55] nor PREG [48] changes BK channel activity in the absence of activating (≥1 μM) levels of $Ca^{2+}{}_i$, i.e., when the channel is gated by intrinsic activity and/or voltage-sensor activation [44, 48]. Second, mutations that render both high-affinity Ca^{2+}-sensing domains in the BK channel cytosolic tail domain (CTD) nonfunctional abolish EtOH action on BK channels [55]. In particular, inhibition of homomeric slo1 channels by EtOH requires Ca^{2+}-activation *via* the high-affinity Ca^{2+} site located in the RCK1 [55]. Likewise, CTD deletion [48] or rendering the RCK1 Ca^{2+}-recognition site nonfunctional *via* the D362A, D367A substitutions abolishes PREG inhibition of cbv1 channel activity [48]. Lastly, both EtOH [44] and PREG (Figure 3D) target Ca^{2+}-driven parameters of BK channel gating to modify activity. Under similar recording conditions to those used in the present study, EtOH has been found to inhibit BK channels that include β1 subunits [44]. While a minor decrease in K_d is caused by exposure of these heteromers to EtOH, this increase in Ca^{2+} apparent affinity is overridden by the EtOH-induced decrease in allosteric coupling of Ca^{2+}-binding to (i) intrinsic gating (i.e., a decrease in parameter C) and (ii) voltage-sensor activation (i.e., a decrease in parameter E) without significant modification of any other voltage-dependent

parameter of gating [44]. The current data showed that cbv1 activity was decreased by PREG despite the fact that K_d was decreased. Therefore, as previously revealed for EtOH, PREG-induced disruption of allosteric coupling to Ca^{2+}-gating is the mechanism leading to the overall decrease in channel activity observed when the channel is exposed to PREG. Indeed, our data have revealed that allosteric coupling between Ca^{2+}-binding and intrinsic channel activity (parameter C) is reduced by PREG (Figure 3D), this action being a key contributor to PREG inhibition of BK channels.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by IACUC of the University of Tennessee Health Science Center, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Author contributions

KN and AD conceived the research. KN, AS, LM, and AB conducted experiments. KN and AS analyzed the data. KN, AS, AB, and AD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adolescent alcohol and nicotine exposure alters the adult response to alcohol use

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Adolescence through young adulthood is a unique period of neuronal development and maturation. Numerous agents can alter this process, resulting in long-term neurological and biological consequences. In the clinical literature, it is frequently reported that adolescent alcohol consumption increases the propensity to develop addictions, including alcohol use disorder (AUD), during adulthood. A general limitation of both clinical and human pre-clinical adolescent alcohol research is the high rate of co-using/abusing more than one drug during adolescence, such as co-using/abusing alcohol with nicotine. A primary goal of basic research is elucidating neuroadaptations produced by adolescent alcohol exposure/consumption that promote alcohol and other drug self-administration in adulthood. The long-term goal is to develop pharmacotherapeutics for the prevention or amelioration of these neuroadaptations. This review will focus on studies that have examined the effects of adolescent alcohol and nicotine exposure on adult alcohol consumption, the hypersensitivity of the mesolimbic dopaminergic system, and enhanced responses not only to alcohol but also to nicotine during adulthood. Again, the long-term goal is to identify potential cholinergic agents to prevent or ameliorate the consequences of, peri-adolescent alcohol abuse.

KEYWORDS

adolescence, alcohol, nicotine, cholinergic system, co-abuse

Human research examining adolescent alcohol consumption

Adolescence is a period in which humans begin to use illicit and age-restricted drugs. Although adolescent drinking has decreased from 2002 to 2021, there is still a high prevalence of adolescent alcohol drinking in the United States [1]. This includes 58% of 12th graders reporting the use of alcohol within the past year and 28% of which engaged in binge drinking within the previous 2 weeks (i.e., >5 or more consecutive

drinks per drinking episode) [2, 3]. Binge drinking is defined by the National Institute on Alcohol Abuse and Alcoholism as 4+/5+ drinks for women/men per occasion respectively (achieving blood alcohol concentrations (BACs) of 0.08 g/dL = 80 mg/dL) [4]. Binge drinking is exaggerated in US young adults since the average binge episode consists of 9.5 drinks/occasion [5, 6].

A recent trend in adolescent/young adult drinking is that the initiation of binge drinking has become progressively earlier, and a sharp increase in the overall rate of binge drinking during the transition from late adolescent/young adulthood into adulthood [7]. Moreover, there has been a focus on studying the effects of high-intensity and extreme-intensity binge drinking in adolescents [8–10]. A longitudinal study comparing US alcohol consumption from 2005 to 2015 indicated consistent levels of binge drinking in 18 year-olds (approximately 20%) [11]. There were also significant rates of high- (approximately 10%) and extreme-intensity (approximately 5%) binge drinking among these US 18 year-olds [11]. In young adults, recent data have indicated that roughly 30% report binge drinking, 11% report high-intensity binge drinking, and 5% report extreme-intensity binge drinking [11].

Alcohol consumption during adolescence is associated with several deleterious consequences. For example, adolescents and young adults display, relative to their adult counterparts, heavier drinking bouts, arrests for driving with ability impaired, and an increased number of arrests for driving while impaired, and an increased rate of alcohol dependence during adulthood significantly associated with the age of the first alcohol drink and the number of binge alcohol drinking episodes during adolescence [12, 13]. The adolescent brain appears to be more susceptible to the effects of binge alcohol consumption than the adult brain [14, 15], and neuroadaptations produced by adolescent alcohol drinking are thought to underlie an increased rate of alcohol misuse in adults suffering from AUDs [16].

Epidemiological studies indicate that individuals who initiate alcohol use before age 15 are 1.3–1.6 times more likely to suffer from an AUD [17]. Additionally, having a family history that is positive [positive (FHP)] for an AUD combined with the initiation of alcohol consumption during adolescence dramatically increases the risk of adult alcohol dependence [18, 19]. A family history of alcoholism also significantly increases observed alterations in white matter integrity (fractional anisotropy) of adolescent binge-drinking subjects [19]. Overall, the clinical evidence consistently indicates that alterations in the central nervous system (CNS) produced by adolescent alcohol consumption are enhanced in individuals with a family history of alcoholism.

A major caveat concerning the interpretation of clinical data on the influence of adolescent alcohol consumption on adult alcohol consumption and the development of an AUD, was the difficulty of adult subjects in recalling their alcohol consumption during adolescence. Given long-term alcohol use and misuse can dramatically affect memory and recall, the accuracy of such first-person accounts have been called into question [20]. However, several recent longitudinal studies have provided clinical support that adolescent alcohol consumption increases the likelihood of adult alcohol consumption or AUD. In a Swedish military conscript study, young adults consuming high-intensity levels of alcohol (8.6 g/day) displayed an increase in alcohol consumption during later adulthood and developed higher rates of developing an AUD [21]. Specifically, the total consumed dose of alcohol during young adulthood increased the future risk for developing an AUD, but a pattern of heavy episodic drinking (high-intensity drinking) significantly increased the later risks for developing an AUD and cirrhosis of the liver [21]. A parallel longitudinal study examining Norwegian and Australian adolescents [22] reported that adolescent alcohol drinking was associated with an increase in adult alcohol consumption and the rate for developing an AUD. Furthermore, the study indicated that interfering with early adolescent alcohol consumption has a protective effect on drinking patterns during late adolescence and adulthood [22]. Overall, recent longitudinal studies have replicated the initial longitudinal study's findings on the deleterious effect of adolescent alcohol consumption on the rate of AUD during adulthood [23–25].

Adolescent and adult alcohol consumption in rodents

The effect of voluntary alcohol consumption during adolescence on adult alcohol consumption

Pre-clinical studies use rodent models to investigate the effect of adolescent alcohol (ethanol) exposure on subsequent neurobiological alterations that contribute to observed changes in behavior related to alcohol drinking in adulthood. The animals are typically exposed to alcohol between early adolescence to late adolescence–emerging adulthood (i.e., postnatal day (PND) 28–65; Table 1), which corresponds with 13–25 years of age in humans [14, 26, 27].

Overall, the field has developed several paradigms to assess rodent drinking, expose animals to binge-like levels of ethanol intake, and/or establish the level of motivation to self-administer ethanol (i.e., operant responding/behavior to obtain alcohol). In typical voluntary free-choice models, animals are allowed access to 2- or 3- bottles with a choice between ethanol or water, or multiple solutions, and animals can freely consume fluid over a 24 h period. Using a slightly different approach, an intermittent drinking paradigm involves alternating periods of access to alcohol with

TABLE 1 Rat and human ages.

Rat ages [Post-Natal Days (PNDs)]

PNDs 1–7	PNDs 8–21	PND 21	18–22	22–27	28–42	43–60	61–75	76–90	90+
3rd Trimester	Infant	Weaning	Childhood	Juvenile	Adolescence	Peri-Adolescence	Emerging Adult	Young Adult	Adult
Human Ages (Years)									
−0.25-0.0 Years	0–2 Years	0.5–2 Years	2 to 6	7–12	13–18	18–21	21–24	25–28	28–50s
					Puberty				
							Adapted from (Bell et al., 2014)		

periods of imposed abstinence to establish binge-like intake in outbred rodents [28]. Studies measuring voluntary ethanol consumption in adolescent non-selected (outbred) rats have produced variable findings. There have been reports that voluntary ethanol drinking during adolescence (PND 28 or PND 31) and continued into adulthood (PND 69–70 or PND 71) had no effect on adult ethanol consumption [29, 30]. On the other hand, Amodeo et al. [31] found that adolescent animals (PND 26–59) exposed to an intermittent voluntary ethanol paradigm, during a period of social isolation, exhibited an increase in operant "appetitive" but not "consummatory" behavior related to ethanol intake during adulthood. Interestingly, after separating the adolescent rats into low (average of 0.29 g/kg/30 min exposure) and high (average of 0.65 g/kg) voluntary ethanol consumption during adolescence, there was a significant increase in adult consumption of ethanol in the 'high' adolescent ethanol drinkers [31].

Adulteration of an ethanol solution using a sweetener (i.e., sucrose or saccharin) enhanced voluntary ethanol consumption during adolescence (PND 29–54; PND 28–42, respectively) [32, 33] and produced biologically relevant blood ethanol concentrations (BEC) as well as enhanced consumption of the same sweetened ethanol solution in adult non-selected rats, respectively. However, this is not always the case. Gilpin et al. [34] reported that operant voluntary binge ethanol consumption in adolescence (PND 28–42) resulted in pharmacologically relevant binge blood ethanol concentrations BAC levels (≥80 mg/dL); however, operant voluntary binge ethanol consumption in adolescence did not alter adult operant self-administration of sweetened ethanol solution or unsweetened ethanol solution in male Wistar rats.

Adolescent voluntary drinking studies using mice have also reported mixed findings on subsequent adult ethanol drinking. An early study using C57BL/6J mice found that adolescent ethanol consumption (beginning at weaning: 3 weeks of age; PND~21) was associated with increased ethanol consumption in adulthood [35]. Nevertheless, voluntary ethanol consumption starting at 5 weeks of age (PND 35) in BALB/cByJ mice also exhibited a greater ethanol preference in adulthood [36]. In contrast, other studies did not observe the same increase in adult consumption following adolescent ethanol exposure (starting at ~PND 21 or ~PND 35) in C57BL/6J [37].

Studies utilizing the murine model have demonstrated that "intermittent-type access to ethanol during adolescence can produce pharmacologically relevant BECs and facilitate adult ethanol intake, but the findings seem to be strain dependent [38, 39]. For example, utilizing the scheduled high ethanol consumption (SHAC) [40] binge procedure produces adolescent BECs of >80 mg/dL in male and female C57BL/6J mice [38]. Moreover, these authors reported that adolescent females were more vulnerable to, i.e., displayed more of, this effect than males [38]. Researchers using the drinking-in-the-dark (DID) binge drinking model, where animals have access to ethanol for 2 or 4 h and 3 or 4 h into the dark cycle, have also reported that adolescent ethanol exposure (PND 28–42) in C57BL/6J, mice produced significantly higher ethanol consumption in adulthood, a finding that was not observed DBA/2J mouse strain [41]. Adult ethanol consumption was also enhanced in C57BL/6J mice following adolescent access [PND 28–36 to low concentration of ethanol (5%; 20 mg/dL BEC) during a modified DID exposure, whereas adolescent DID ethanol consumption (20%, 20 mg/dL BEC)] in DBA/2J did not alter ethanol consumption in adulthood [42]. These findings would suggest that, while the DID paradigm can produce binge-like ethanol intake in adolescent animals, genetic background likely plays a role as well. That is, genetic factors that have established the C57BL/6J mouse line as a high ethanol-drinking model, likely contributed to the observed increase in ethanol intake in adulthood as the effect was present even when adolescent animals were exposed to low concentrations (non-binge levels: BEC <80 mg/dL) [42].

Collectively, the literature suggests that under certain conditions, voluntary ethanol consumption during adolescence can produce enhanced ethanol consumption in adulthood. In some cases, alterations in drinking behavior, due to adolescent ethanol exposure, may be mediated by sex-of-animal effect and/or genotype, however, obtaining biologically relevant blood ethanol levels, (BEC >80 mg/dL) appears to be a critical factor. Towner and Varlinskaya [39] reported that one-third

of voluntary adolescent ethanol consumption in rodents resulted in a subsequent increase in adult ethanol consumption and that pharmacological levels of ethanol during adolescence may have to be well above a BEC of 80 mg/dL (e.g., 100–200 mg/dL) to increase subsequent adult ethanol consumption. Thus, DID, SHAC, and forced ethanol (e.g., IP, IG, vapor inhalation) procedures in adolescent rodents tend to result in higher BECs and more consistent increases in adult ethanol consumption [39].

Voluntary alcohol consumption during adolescence effects on adult alcohol consumption in alcohol-preferring rodent models

Selective breeding for high ethanol intake has produced multiple rat lines that voluntarily consume pharmacologically relevant levels of ethanol under 24 h free-access drinking conditions [e.g., alcohol-preferring (P) and Alko Alcohol (AA)] [c.f. 43, 44]. Such lines have been utilized as a powerful tool to examine the influence of genetic background contributing to the behavioral and neurobiological components of AUDs.

Alcohol preferring (P) rat line

The P rat line has demonstrated reliable consumption of biologically relevant levels of ethanol from post-natal day (PND) 7 until death) [45]. Twenty-four free-access drinking access during adolescence results in binge-level of ethanol intake (>80 mg%) in P rats [8]. In P rats, voluntary ethanol consumption during adolescence can alter ethanol-related behaviors during adulthood [46–49]. For example, 24 h free-access ethanol drinking during PND 22–71 increased ethanol consumption during ethanol re-exposure in adulthood (PND 99) compared to control animals [47]. Adolescent (PND 28–60) voluntary ethanol consumption in P rats (average intake of 6.3 g/kg/day) has also been shown to increase the rate of acquisition of operant ethanol self-administration during adulthood (PND 90) compared to animals exposed to the same regimen completely in adulthood (PND 137–169; testing started at PND 199) [48, 49]. Ethanol consumption during adolescence produced resistance to extinction, increased the expression of both context- and ethanol primed-induced ethanol seeking, enhanced relapse drinking, and significantly increased breakpoint in adulthood [48, 50]. Moreover, these effects on operant behavior were observed during the second cycle of testing for extinction, seeking, and relapse, suggesting that voluntary adolescent ethanol consumption can produce persistent effects on ethanol-related behaviors well into adulthood [48]. It is also important to mention that these findings were specific to ethanol as a control study using the same paradigm, but exposing animals instead to saccharin during adolescence, did not alter adult saccharin self-administration, saccharin extinction learning, relapse, or breakpoint [50].

Alko, alcohol (AA) rat line

The AA rat line also readily consumes pharmacologically relevant levels of ethanol (5–8 g/kg) in 24 h with BECs as high as 50 mg% [51–53]. A recent report found that voluntary adolescent ethanol consumption (starting at PND 42 and continuing for 6 weeks) in female AA rats did not increase subsequent ethanol drinking in adulthood, and by extension did not increase ethanol preference [54]. Although the AA rats consumed pharmacologically relevant levels of ethanol during adolescence, animals failed to establish binge-like (>80 mg%) BECs, which may have been a reason as to why the authors failed to observe increased ethanol consumption in adulthood [54]. Further, the timing of adolescent exposure (i.e., mid-late adolescence) may have also been another factor. Previous work suggests that early-mid (PND 28–45) adolescent exposure induces more severe neuroadaptations than mid-late ethanol exposure [26, 54]. Regardless, the data supports the notion that, even in alcohol-preferring rat models, attaining high (i.e., binge-like or higher) BECs during adolescence appears to be important for subsequent increases in adult ethanol drinking.

Adolescent intermittent ethanol (AIE) exposure effects adult alcohol consumption

Vapor exposure of alcohol in adolescence

The adolescent intermittent ethanol (AIE) exposure model is an experimenter-administered binge model (e.g., ethanol vapor, intragastric (IG), intraperitoneal (IP) injection), used to produce consistent binge BEC levels in rodents that do not readily consume ethanol [cf., 6]. The BEC levels achieved with AIE are approximately 160 mg% (i.e., 0.16 g/dL) or greater in rats and mice [55–60]. However, studies utilizing AIE exposure have reported mixed findings. High level AIE vapor exposure (PND 28–42) that established BECs in excess of 300 mg% produced an increase in operant responding for (maintenance) and a resistance to extinction of operant self-administration during adulthood (PND 65–90) in Long-Evans [61]. However, utilizing an identical AIE regimen and rat strain, Nentwig et al. [62] failed to observe similar AIE-induced alterations to adult 2-bottle choice voluntary ethanol consumption or operant self-administration behaviors. Studies examining AIE in mice are also mixed. For instance, AIE (PND 28–42) vapor exposure increased ethanol consumption in adult male C57BL/6J mice

following short-term abstinence during late adolescence and early adulthood (PND 50–76) and following protracted abstinence (PND 70–97) in adulthood, but did not alter consumption in female mice [63] indicating that potential sex differences may be at play in vulnerability to the effects of AIE vapor exposure.

Systemic administration of alcohol in adolescence

Several studies have utilized the systemic route of administration (i.e., IP injection or IG gavage) of ethanol during adolescence to examine the long-term effects on ethanol drinking in adulthood. Ethanol administered via the IP or IG routes has been shown to produce equivalent ethanol exposure between subjects that is not approximated in consumption/drinking paradigms, and reliable, dose-dependent, BECs. However, much like the research discussed thus far, the findings have been mixed and there seem to be dose- and sex-dependent, as well as age-related variables that underlie the enduring behavioral effects observed using these techniques.

An early study by Gilpin et al. [34] reported that adult animals exhibited a conditioned taste aversion to sweetened solutions and consumed significantly less sweetened and unsweetened ethanol following adolescent ethanol injections (PND 28–42; 2 g/kg/IP) compared to control animals. In contrast, administration of a lower dose of ethanol (1.5 g/kg/IP; PND 30–50) enhanced consumption of a sweetened ethanol solution during limited access testing in female adult rats (PND 65–80), whereas reducing the dose by half (0.75 g/kg/IP) did not [64]. A similar finding was reported using a higher dose of ethanol (3 g/kg/IP; PND 25–38) in that, adult animals (PND 60) exhibited an increase in ethanol consumption, which produced BECs that were not pharmacologically relevant (<30 mg%) [65]. In a similar experiment, ethanol administration of the same dose of ethanol (3 g/kg/IP) during early-mid adolescence (PND 30–43), but not late adolescence (PND 45–58), increased operant self-administration of ethanol as well as ethanol consumption when animals were provided both free and intermittent access ethanol in young adulthood [66].

Varying effects have also been reported in studies utilizing adolescent IG administration of ethanol. Maldonado-Devincci et al. [67] indicated IG administration of 1.5, 3.0, or 5.0 g/kg ethanol in 4 days intervals (PND 28–31, PND 35–38, and PND 43–45) increased ethanol consumption in young adulthood (PND 60–69) in both male and female Sprague-Dawley (SD) rats, an effect more prominent in male compared to female animals. Intermittent IG ethanol exposure (PND 28–48; 2 days on/2 days off) treatment in male and female P rats resulted in increased adult ethanol consumption (PND 90+) during both operant acquisition and relapse drinking conditions [68, 69]. In contrast, repeated gavage (every 8 h for 2 days: 6 treatments total) during adolescence (PND 30–32) decreased ethanol consumption in adulthood in Sprague Dawley rats [70]. The differences in behavioral data between these studies may be due to the gavage procedure, the length of time animals received exposure, and the strain of rats (P vs. SD rats).

Adolescent alcohol consumption and nicotine use—clinical data

Co-use/abuse is common among adolescents and young adults [71, 72]. A limitation of research is the ability to account for co-use/abuse of more than one drug during the window of adolescence and the effect this may have on the development of drug-related issues later in life. Adolescent alcohol drinking has been linked to increased adulthood use of opioids, cannabis, nicotine, and other drugs of abuse [73, 74]. Specifically for nicotine, adolescent binge drinking enhances the likelihood of smoking during adolescence by 88% as well as during adulthood, while individuals who do not binge drink during this period have lower smoking rates during adolescence and adulthood [74, 75]. Moreover, adolescents who use nicotine have higher rates of AUDs than their non-smoking counterparts [75, 76]. Simultaneous alcohol and tobacco use during early adolescence (age: ~12 years old) promoted an escalation of drug intake during late adolescence and was associated with a higher rate of adult drug addiction, AUD, and co-substance drug addiction [77].

The use of non-combustible nicotine via electronic delivery (i.e., electronic cigarettes [e-cigarettes] or vaping pens) has become a popular alternative to cigarette smoking, especially among adolescents over the last decade [78]. In 2016, 38% of all high school students stated they had tried e-cigarettes, a rate comparable to alcohol usage in the same demographic [79]. Initiation of e-cigarette use during adolescence is associated with a greater prevalence of traditional nicotine use later in life (cf. [6]) and may promote traditional nicotine use in individuals who would not have initiated use otherwise [80]. Adolescents who use e-cigarettes are 6.5 times more likely to consume alcohol (including bouts of binge drinking), compared to those who do not use e-cigarettes [81]. The liquid nicotine solutions for e-cigarettes can contain 92%–104% more nicotine than stated by the manufacturers (cf. [81]). In addition, individuals who "vape" also receive a significant dose of alcohol as the range of alcohol concentration in liquid nicotine solutions ranges from 0.4%–23.5%, with the most popular brands ranging from 10% to 18% [82]. The rate of absorption of alcohol through the "vaping"'route is extremely high, and alcohol metabolites can be detected in individuals actively "vaping" [82]. With "hacked" e-cigarette systems, the rate of alcohol, acetaldehyde, and aldehyde intake can increase in magnitude, and reach detectable levels within the brain without

reaching detectable levels in the periphery [83]. Replicate findings have indicated that alcohol and nicotine co-use/abuse during adolescence enhances adult drug dependency compared to the consumption of only alcohol or nicotine during adolescence (cf. [84]).

Modeling adolescent alcohol consumption and nicotine use–pre-clinical data

For several years, rodent experiments have sought to model ethanol/nicotine use/co-use during the developmental period of adolescence in the clinical population to determine the behavioral and neurobiological effects of these compounds that contribute to drug addiction both during adolescence and later in life. Several techniques have been used to investigate the effects of adolescent nicotine exposure on ethanol reward in adolescence and adulthood, such as oral nicotine access, injections (IP and subcutaneous: SC), intravenous (IV) self-administration, and nicotine vapor exposure. Overall, the findings are mixed as some rodent studies have demonstrated that adolescent nicotine exposure can increase ethanol consumption, while other studies report no effect. The discrepancy between studies seems to be related to when in adolescence (i.e., early, mid, or late) the animals are exposed to nicotine as well as the species used (mouse versus rat). For instance, oral nicotine exposure (200 μg/mL/22 h) during early-mid adolescence (~PND 35–44) increases binge-like ethanol consumption and BECs in mid adolescence (~PND 45–48) in female C57BL/6J mice [85, 86]. Oral nicotine consumption (30 μg/mL) using the DID model during late adolescence (PND 42–56) did not affect subsequent binge-like ethanol consumption in early adulthood in male C57BL/6J mice (PND 56–78) [87]. Repeated systemic administration of nicotine (0.4 mg/IP) during adolescence (PND 28–PND 42) induced long-lasting increases in adult ethanol self-administration, while adult (PND 60–74) nicotine administration did not alter subsequent adult ethanol self-administration in male Long-Evans rats [88].

Chronic continuous nicotine exposure (subcutaneous 21 day time-release pellets) during adolescence (PND 35–56) did not increase ethanol intake in SD rats during young adulthood (PND 53 -PND 74) [89]. Peripheral administration of nicotine (0.4 mg/kg/IP) administered in early adolescence (PND 28–32), prior to ethanol operant training, and then re-administered 2 h after ethanol training session during early-late adolescence (PND 33–56) did not alter self-administration or motivation (i.e., breakpoint) of sweetened ethanol [90]. Recently, Ruffolo et al. [91] examined the effects of vaporized JUUL e-cigarette mint flavored 5% nicotine e-liquid pods on adult ethanol consumption. Their findings indicated that adolescent nicotine vapor exposure (PND 30–46), voluntary ethanol consumption alone, and combination with nicotine exposure (i.e., nicotine vapor exposure, followed by voluntary ethanol consumption) did not alter ethanol intake or preference in adult SD [91]. IG adolescent ethanol exposure (PND 30–32; every 8 h for 2 days: 6 treatments total) also failed to alter ethanol and nicotine co-use during adulthood in SD rats [70].

However, several studies have indicated that co-exposure to ethanol and nicotine during adolescence results in distinct behavioral and neurochemical effects in adulthood that are not observed following adolescent exposure to ethanol or nicotine alone [92–94]. The effects of simultaneous exposure to ethanol and nicotine during adolescence have been reported to increase memory/learning deficits and enhance anxiety-like and drug-seeking behaviors in mice [93, 95, 96]. A recent study demonstrated that simultaneous IV self-administration of ethanol + nicotine during adolescence (PND 32–41) enhances ethanol reinforcement and intake in late adolescence and emerging adulthood in male rats (PND 48–65) [97]. A similar effect was not observed in male nor female rats when the same exposure and testing regimen occurred solely in adulthood (PND 90–99) [97]. Conversely, some studies have indicated that ethanol/nicotine co-exposure during adolescence (PND 30–45) results in certain adult alterations that parallel single drug exposure [98]. Taken together, these pre-clinical studies provide evidence that the effect of adolescent nicotine exposure or adolescent co-exposure to ethanol and nicotine on behavior is fairly complex, and further studies will be needed to determine how exposure, alone or in combination, during this developmental window affects use/abuse liability later in life.

The effect of alcohol during adolescence on dopamine function

The mesocorticolimbic (MCL) dopaminergic (DAnergic) system is involved in processing the rewarding effects of natural reinforcers and drugs of abuse, and it undergoes significant developmental changes during adolescence [14]. For example, during mid-to-late adolescence, DA neurons in the ventral tegmental (VTA), that project to the nucleus accumbens (Acb), are firing at their highest rate, suggesting a pattern of DA overproduction and increased DA receptor expression throughout the circuit, which declines in adulthood [65, 99, 100]. Philpot et al. [101] provided further evidence that basal DA levels in Acb increased through developmental stages of preadolescent (PND 25; lowest DA levels), early adolescent (PND 35), and late adolescent (PND 45; highest DA levels) with a decline in young adulthood (PND 60).

Drugs of abuse effects on DA release are typically observed in the Acb. The Acb is divided into 2 distinct anatomical and functional structures (i.e., shell [AcbSh] and the core [AcbC]), and each plays a different role in reward and motivation. Reports

have demonstrated that ethanol, nicotine, and other drugs of abuse preferentially increase DA release in AcbSh compared to AcbC [102]. Moreover, AcbSh receives more DA projections from the VTA than the AcbC [103]. The AcbSh plays a critical role in the reinforcing effects of rewards and spatial/contextual drug-seeking behavior, while AcbC is involved in the motivation to seek rewards and mediating cue-induced drug-seeking behavior [104].

The effects of adolescent ethanol exposure on DA release have been examined in both the AcbSh and AcbC. A consistent finding in the pre-clinical literature is that ethanol exposure during adolescence produces a persistent hyper-dopaminergic state, and this is observed in the AcbSh [65, 105–108]. Pascual et al. [65] reported that basal extracellular DA levels in the AcbSh, following AIE injections (3 g/kg/IP) in adolescence (exposure: PND 25–38; DA collection: PND 41) were higher than if the animals were animals pre-treated with ethanol during adulthood. Moreover, adolescent AIE exposure resulted in a higher expression of D1 and D2 receptors in adolescents compared to adult-exposed animals lending support to previous studies [65]. In a subsequent study, 4 days of repeated administration of low to moderate ethanol (0.5, 1.0, or 2.0 g/kg/IP) with the test days on PND 25 (preadolescent), PND 35 (early adolescent), and PND 45 (late adolescent) increased DA levels in the Acb in all the stages of adolescence [101]. However, the ethanol's peak effects on DA decreased during pre-adolescence and early adolescence, following a challenge dose of ethanol, but was not altered in late adolescence or young adulthood [101]. These findings indicated that PND 35 and 45 appear to be a key developmental transition periods to the neuroadaptation of effects of repeated ethanol exposure [101]. In contrast to [65], this study found that young adulthood (PND 60) ethanol exposure also increases DA levels in Acb [101]. This difference between studies may be due to different ethanol exposure methods and doses of ethanol.

In another study, moderate ethanol exposure (1 g/kg, every other day) in adolescents (PND 30–50) reduced DA release in the AcbC following a challenge dose during adulthood, with the reduction becoming less robust as the abstinence period (7, 14, 49 days) between adolescent exposure and challenged ethanol dose test increased [109]. The authors finding suggested ethanol exposure starting during early adolescence, not during adulthood (PND 60–80), resulted in a decline in the responsiveness of DAnergic neurons in the AcbC to ethanol [109]. Collectively, these studies demonstrate adolescent ethanol exposure induces age-dependent effects on the MCL DAnergic system that may contribute to the increased risk of AUDs in adulthood.

Ethanol administration in adolescence has also been shown to increase basal DA levels in the AcbSh during adulthood [105, 106]. Injections of ethanol (0.75 g/kg/IP) for 21 consecutive days during adolescence (PND 30–50) produced a persistent increase in basal DA in the shell region of the Acb during adulthood (PND 70) compared to saline pre-treatment [106]. Further, the observed increase in DA was likely due to increases in efflux as there were no changes in DA reuptake [106]. In P rats, 24 h continuous voluntary ethanol consumption from PND 30 to PND 60 produced a prolonged increase (i.e., 2 h long) in the extracellular DA levels and increased DA uptake in Acb following ethanol challenge (2.5 g/kg/IP) in adulthood compared with saline-challenge [105]. The observed differences in the AIE induced increase in DA uptake between studies are possibly due to the use of different rat strains (P rats vs. SD). Research has found that P rats have an abnormal innate DA profile with lower DA and DA metabolites than their control, the non-preferring (NP) rats, which contributes to P rats' high ethanol drinking behavior [110].

The VTA is a heterogeneous structure. Intracranial self-administration studies, which elucidate specific neuroanatomical sites that support drug self-administration, have shown that posterior VTA (pVTA), but not the anterior VTA (aVTA), is a neuroanatomical site mediating the reinforcing actions of drug reinforcement (i.e., ethanol, cocaine, nicotine, delta9 tetrahydrocannabinol [111–115]). Furthermore, the reinforcing effects of pVTA require the activation of DAnergic neurons [116, 117]. Adolescent ethanol exposure has been shown to sensitize DA neurons in the pVTA as challenge doses of ethanol more readily enhance DA levels in adulthood [107, 108]. For example, P rats provided free access to ethanol during adolescence (PND 30–60), and Wistar exposed to an AIE regimen (PND 28–48), exhibited a leftward and upward shift of the dose-response curve when receiving microinjections of ethanol directly into the pVTA during adulthood. Together, these findings provide some evidence that ethanol exposure during adolescence may produce a hyper-dopaminergic system of ethanol that may, in part, underlie the biological basis for enhanced adult ethanol consumption.

The effect of alcohol and nicotine on dopamine function

Ethanol and nicotine share some common mechanisms of action. For example, the reinforcing effects of nicotine are modulated via stimulation of nicotinic acetylcholine receptors (nAChR) receptors within the VTA [118, 119], and ethanol reinforcing effects are partially modulated by nAChR receptors [120–122]. For example, the antagonism of nAChR with mecamylamine, a non-selective nAChR antagonist, can reduce ethanol intake [123, 124] and nicotine-stimulated ethanol drinking [125, 126]. A limited number of pre-clinical studies have focused on the manner in which neuroadaptations that occur due to ethanol exposure during adolescence contribute to the co-use/abuse of drugs later in life. Recently, Waeiss et al. [127] observed that voluntary adolescent ethanol consumption (PND 30–60) increases the sensitivity (leftward shift in the dose-response curve) to the effects of nicotine within the MCL reward circuit (pVTA nicotine microinjection, DA release in AcbSh;

125). To date, however, the majority of studies have featured adult animals.

Combined peripheral injections of ethanol and nicotine, as well as a combined administration of low doses of ethanol peripherally and nicotine centrally (into VTA), have been shown to have an addictive effect on DA release in AcbSh of adult male Wistar rats [128, 129]. In adult P rats, acute exposure to ethanol and additional drugs (nicotine, cocaine, etc) results in unique, synergistic, or additive effects in various brain structures (e.g. [130, 131]). Chronic simultaneous ethanol and nicotine co-use results in unique adaptations in discrete brain regions that enhance drug reward in adult P rats [132, 133]. For example, microinjections of pharmacologically relevant doses of ethanol or nicotine directly into the pVTA induce DA release in the AcbSh [134]. Co-administration of subthreshold concentrations of ethanol and nicotine combined into the pVTA increases DA and glutamate release in the AcbSh, whereas the same subthreshold concentrations of each drug microinjected alone does not [130]. Furthermore, microinjections of ethanol + nicotine (but not ethanol or nicotine alone) into the pVTA altered the sensitivity of ethanol in the AcbSh and the expression of brain derived neurotrophic factor (BDNF) in the AcbSh (weeks following pVTA microinjections) [127]. Overall, the data indicate that co-administration of ethanol + nicotine differentially activates the mesolimbic DA system, which is not observed following the administration of the compounds individually.

Potential pharmacotherapies to mitigate the effects of binge-like ethanol consumption during adolescence as it pertains to adult ethanol consumption

One of the primary goals of the pre-clinical adolescent ethanol research, is to develop useful strategies and/or pharmacotherapeutic treatments that are capable of counteracting the deleterious effects of binge-like ethanol consumption/exposure during adolescence and the subsequent increase in adult ethanol/drug use/abuse that may eventually be utilized to treat the clinical population. So far, the majority of research has focused on describing the biological consequences of adolescent ethanol exposure and there have been limited attempts to assess potential pharmacological interventions to counteract the behavioral and neurobiological consequences of adolescent binge-like ethanol exposure. Simply put, there are two approaches to counter the deleterious effects of binge-like ethanol exposure: reversal or prevention with the majority of effort focused on the latter. The few studies that have focused on "reversing" the effects of adolescent ethanol consumption/exposure have 'focused on "correcting" the biological alterations produced by exposure to ethanol during the adolescent window.

Alterations in the central cholinergic system are implicated in nicotine and ethanol abuse [6, 135]. There are two classifications of cholinergic receptors: nicotinic and muscarinic. Nicotinic acetylcholine receptors (nAChRs) consist of 11 neuronal subunits, which are divided into 8 alpha subunits (*α2-α7*, *α9-α10*) and 3 beta subunits (*β2-β4*). Adolescent ethanol consumption/exposure has been reported to upregulate α7 nicotinic receptors, which are usually homomeric, during adulthood [127, 136]. Evidence also suggests that adolescent ethanol or nicotine exposure alone can lead to subsequent cholinergic dysfunction [6, 15, 137]. For example, choline acetyltransferase (ChAT), a cholinergic marker that is responsible for the biosynthesis of the neurotransmitter acetylcholine, is reduced in several brain regions following adolescent ethanol or adolescent nicotine exposure [6, 15, 137].

Varenicline

This has led to an investigation of the effect of cholinergic compounds on adolescent ethanol exposure induced cholinergic deficits that persist well into adulthood [15, 137]. The FDA-approved smoking cessation aid Varenicline (i.e., Chantix), an α4β2 nAChR partial agonist, has a slower onset and longer duration on DA release compared to nicotine na d can block nicotine's effect on DA release [138]. Varenicline has been reported to reduce ethanol consumption in adolescent C57BL/6J mice, after four 2 day DID sessions at PND 32–33, 36–37, 39–40, and 43–44, with Varenicline administered on the second of these 2 day sessions 30 min prior to ethanol access [139].

Cholinesterase inhibitors

The FDA-approved cholinesterase inhibitors Donepezil and Galantamine administered during peri-adolescence to emerging adulthood (PND 69–72; PND 57–72) can reverse AIE (PND 30–48; PND 25–54) induced alteration or deficits (e.g., reversing AIE decreases in dendritic spine density or the persistent losses of cholinergic neuron markers) observed during adulthood in the hippocampus (i.e., learning and memory) and basal forebrain (i.e., behavioral control, attention, and other executive functions) [59, 137, 140]. Moreover, Galantamine administered during AIE exposure (PND 25–54) prevented the AIE induced deficits in the basal forebrain [137]. Interestingly, although Donepezil and Galantamine are both cholinesterase inhibitors, findings have demonstrated that Galantamine, but not Donepezil, increases the firing activity of DAnergic cells in the VTA [141]. Galantamine effect on DA through its allosteric potentiation of nAChRs [141]. Therefore, some of Galantamine's blocking and reversing effects of AIE may partly be through the DAnergic system.

Galantamine effects also appear to involve the α7 nAChR mediating presynaptic facilitation of glutamate release [141]. In addition, the neuroprotective effects of Donepezil are mediated through α7 nAChR [142, 143]. Glutamatergic projections to MCL are involved in the development of drug-seeking and drug-taking behavior [144]. Moreover, excessive glutamate can induce excitotoxicity and loss of neurons [145]. The α7 nAChRs have a lower affinity for nicotine and are located presynaptically on glutamatergic terminals [146]. Thus, it is suggested that activation of α7 nAChR enhances glutamatergic excitatory drive and may promote DA release after the α4β2 receptors are desensitized [146]. Thus mediating the long-term effects of chronic nicotine exposure [147].

α7 nAChR negative and positive allosteric modulator (NAM and PAM)

Administration of an α7 nAChR negative allosteric modulator (NAM) dehydronorketamine (DHNK) 2 hours before AIE exposure (PND 28–48) prevented the increase of ethanol consumption during acquisition and relapse drinking during adulthood in both male and female P rats [68]. A subsequent study reported that SB-277011-A, an α7 nAChR NAM and a D3 antagonist, could also suppress ethanol consumption during acquisition and relapse drinking in female P rats [69, 148]. In contrast, to α7 nAChR NAMs, activation of the α7 nAChR during adolescence appears to have the opposite effect on ethanol consumption in adulthood. Intermittent adolescent treatment (PND 29, 30, 33, 35, 36, and 37) with the α7 nAChR agonist AR-17779 increased the amount of ethanol consumed during acquisition and relapse during adulthood (PND 90+) in both male and female P rats [68]. Furthermore, co-infusion of α7 nAChR agonist + ethanol into pVTA increased extracellular DA release in AcbSh to a significantly greater extent than either treatment alone in male Wistar rats [69]. In addition, administration of α7 nAChR positive allosteric modulator (PAM) PNU- 120596 followed by low-dose ethanol (gavage, 2 mg/kg) in adolescents (PND 28–48) increased operant beer acquisition, extinction, and relapse drinking in adulthood in female Wistar rats [69]. However, PNU +2 mg/kg ethanol treatment during adolescence did not affect 24-h free-choice ethanol drinking of adult male Wistar rats [69].

Interestingly, AIE (PND 28–48) and peri-adolescent (PND 30–50) ethanol consumption did not alter glutamate release [108] or glutamate transporters [147] in Acb, respectively. However, adolescent nicotine consumption and ethanol + nicotine intake reduced glutamate transporter-1 (GLT-1), which reuptakes 90% of glutamate [145]. Therefore, further research is warranted to determine mechanisms that the α7 nAChR involvement with the glutamatergic system.

Bupropion, Lobeline, and Cytisine

Other nicotinic cholinergic compounds have also been shown to have some effectiveness in reducing ethanol consumption in pre-clinical studies. For example, Bupropion is FDA-approved for smoking cessation as well as depression and seasonal affective disorders. It is an antagonist for α3β2, α4β2, and α7 nAChRs, with Bupropion being ~50 and 12 times more effective in blocking α3β2 and α4β2 than α7nAChRs [149]. Bupropion is also a dual norepinephrine and DA reuptake inhibitor (cf., [149]). Bupropion effects on ethanol intake have varied with no effects on limited access (2 h) to ethanol in P rats [150] to reducing DID ethanol (2 h) intake in C57BL/J mice [151].

Lobeline is a non-selective antagonist nAChRs that can inhibit nicotine and ethanol DA release [152]. Lobeline also inhibits DA and vesicular monoamine transporters. Cytisine is partial agonist for α4β2 nAChR and a full agonist at β4 and α7 nAChR. Nicotine- and ethanol-induced extracellular DA release can be reduced by Cytisine [cf., 154]. Both compounds have been reported to reduce ethanol consumption in high-alcohol drinking models (i.e., C57BL/6J mice and high alcohol drinking line 2 rats [152–154]) as well as reduce ethanol-induced DA release [155]. However, no research has examined whether Bupropion, Lobeline, or Cytisine would effectively block or reverse the behavioral and neurobiological changes observed in adulthood following adolescent ethanol or ethanol+ nicotine exposure.

Collectively, these studies provide evidence that treatments targeting the nicotinic cholinergic system may represent viable pharmacotherapeutic compounds to 'reverse' the effects of adolescent ethanol exposure on ethanol-related neuropathology and drinking behaviors observed during adulthood. Moreover, it will be interesting to extend this line of research to investigate other nicotinic cholinergic compounds as well as examine the utility of these compounds to reverse the additive effects of adolescent ethanol + nicotine exposure on ethanol and/or co-use/abuse in adulthood.

Conclusion

Pre-clinical and clinical research has indicated that adolescent alcohol and/or nicotine consumption/exposure can promote alcohol consumption during adulthood. The likelihood of observing this effect in pre-clinical research is increased if adolescent rats are exposed to biologically relevant levels of ethanol, with appropriate drinking protocols in adulthood, which produce pharmacologically relevant levels of ethanol intake. Recent experiments have indicated potential pharmacological targets that can reverse or prevent some of the persistent deleterious behavioral and neurobiological changes observed during adulthood following adolescent ethanol consumption/exposure. However, there is still a

need to elucidate the mechanism and neural substrates that may contribute to the effectiveness of these cholinergic compounds. Pre-clinical research needs to be conducted to determine if the FDA-approved Donepezil, Galantamine, and Bupropion can attenuate adolescent alcohol and/or nicotine consumption/exposure ability to increase the risk of alcohol consumption during adulthood. Moreover, further research is still warranted to better understand adolescent co-use/abuse effects during adulthood, with a goal of developing novel efficacious pharmacotherapies to treat AUDs and co-use/abuse.

Author contributions

SH drafted the manuscript. SH, RW, GD, EE, RB, and ZR provided a critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ZR, SH, RW, and RB have applied for a provisional patent titled 'Use of Alpha-7 Nicotinic Receptor Negative Allosteric Modulators reduce the deleterious effects of adolescent alcohol consumption during adulthood' with the US and EU patent office.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alcohol induced behavioral and immune perturbations are attenuated by activation of CB2 cannabinoid receptors

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The endocannabinoidome (eCBome) is the expanded endocannabinoid system (ECS) and studies show that there is a link between this system and how it modulates alcohol induced neuroinflammation. Using conditional knockout (cKO) mice with selective deletion of cannabinoid type 2 receptors (CB2Rs) in dopamine neurons (DAT-*Cnr2*) and in microglia (Cx3Cr1-*Cnr2*), we investigated how CB2Rs modulate behavioral and neuroinflammation induced by alcohol. Behavioral tests including locomotor and wheel running activity, rotarod performance test, and alcohol preference tests were used to evaluate behavioral changes induced by alcohol. Using ELISA assay, we investigated the level of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1α (IL-1α), and interleukin-1β (IL-1β) in the hippocampus of mice. The findings demonstrated that locomotor activity, wheel running, and rotarod performance activities were significantly affected by cell-type specific deletion of CB2Rs in dopamine neurons and microglia. The non-selective CB2R agonist, WIN 55,212-2, reduced alcohol preference in the wild type and cell-type specific CB2R cKO mice. In addition, the result showed that cell-type specific deletion of CB2Rs *per se* and administration of alcohol to CB2R cKO mice increased the expression of proinflammatory cytokines in the hippocampus. These findings suggest the involvement of CB2Rs in modulating behavioral and immune alterations induced by alcohol.

KEYWORDS

eCBome, ECS, alcohol, inflammation, CB2Rs

Introduction

The characterization of additional lipid mediators, enzymes and receptors, has led to the discovery of an expanded endocannabinoid system (ECS) called the endocannabinoidome (eCBome) [1]. The ECS is composed of two canonical cannabinoid receptors (CBRs); cannabinoid type 1 receptors (CB1Rs) and

cannabinoid type 2 receptors (CB2Rs), endocannabinoids (eCBs) and enzymes responsible for the synthesis and degradation of eCBs [2, 3]. While cannabinoids represent a group of substances that share the common property of binding with cannabinoid receptors (CBRs), only two substances, arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol, are considered primary eCBs [4–6]. CB1Rs, which are expressed in the hippocampus, neocortex, cerebellum, and basal ganglia nuclei, are the most abundant GPCRs in the brain [3]. CB2Rs are found in abundance in the periphery and predominantly in organs with immune function [7–9]. Contrary to the previous notion that CB2Rs were absent in brain [9–11], a growing body of evidence now demonstrates CB2R expression in microglia, and neurons in the hippocampus, striatum and brain stem [12, 13]. There has been continuous debate and controversy about the expression of functional neuronal CB2Rs, however, following our discovery of the presence and functional expression of CB2Rs in brain [14–17], other studies have overwhelming confirmed that functional CB2Rs are present in neurons and are regulated by drugs of abuse [18–21].

Chronic alcohol consumption, through abnormal brain circuits, can cause neuronal damage, behavioral alterations, and neuroinflammation that are characterized by an enhanced release of pro-inflammatory cytokines called cytokine storm [22–24]. Recent preclinical reports suggest that enhanced innate immune system signaling increases consumption of alcohol [25]. Studies also indicated that CB2R activation has been shown to inhibit neuroinflammation, attenuate neuronal tissue damage, and drive neurogenesis [26, 27]. We hypothesized that CB2Rs can play a role in preventing alcohol induced behavioral and neuroimmune changes in mice. We addressed this question by investigating the roles of dopamine neuron and microglia CB2Rs using DAT-*Cnr2*, Cx3cr1-*Cnr2* cKO, and wild type (WT) control mice in modulating behavioral and neuroimmune alterations induced by the effects of alcohol.

Materials and methods

Animals

In this study, we employed DAT-*Cnr2* and Cx3Cr1-*Cnr2* cKO mice which are created in our lab [28]. The mice were generated through a breeding approach involving *Cnr2*-floxed mice and DAT-*Cre* and Cx3-*Cre* mice. We confirmed the specific deletion of CB2Rs in dopamine cells and microglia in homozygous cKO mice through genotyping and RNAscope *in situ* hybridization, while no deletion occurred in the WT mice. The experiments were conducted on adult male mice weighing between 20 g and 30 g, all bred in the mouse laboratory at William Paterson University of New Jersey. These mice were kept under controlled conditions, including room temperature (25°C ± 2°C), a 12:12 h light-dark cycle, and *ad libitum* access to food and water. Our study adhered to the guidelines in the Guide for the Care and Use of Laboratory Animals and received approval from the William Paterson University Animal Care and Use Committee (IACUC).

Drugs and administration

Absolute ethanol was purchased from Pharmaco-AAper in Bristol, PA. 8% of the absolute alcohol was mixed with distilled water and administered as 0.8 g/kg dose into the peritoneum (i.p.) at a volume of 10 mL/kg body weight. The non-selective cannabinoid receptor agonist, WIN55,212-2 (WIN), was purchased from Cayman Chemical Co. located in Ann Arbor, MI. After dissolving WIN in a mixture of DMSO, tween 80, and saline in a ratio of 1:2:7, a dosage of 3 mg/kg was administered. The doses of alcohol and WIN were determined based on previous research [21, 28–30]. Both alcohol and WIN were injected i.p. in a volume of 10 mL/kg body weight.

Locomotor activity test

To evaluate total distance travelled in the activity box, the locomotor activity monitoring apparatus (ENV-510: Med Associates Inc., St. Albans, VT, USA) was utilized. Thirty minutes after acute alcohol injection, the animals were placed gently into separate test boxes (measuring 43.2 × 43.2 × 30.5 cm) that were connected to a computer. Total distance traveled by mice was recorded and analyzed over a 10-min period [21]. Prior to the test, the mice were given three consecutive days to freely explore the open field chambers for 10 min each day in order to acclimate to the environment.

Wheel running activity test

The wheel running activity of the mice was observed using a spontaneous wheel-running monitor (Wahmann, Geo. H., Manufacturing Company, Baltimore, MD, USA) after 40 min of acute alcohol administration. Each mouse was placed in the monitor, and their wheel running behavior was tracked using auto-counters, which recorded the total number of revolutions made by each animal during the 10-min testing session [21].

Rota rod performance test

Mice were placed on a stationary rota rod (AccuRotor Rotarod, AccuScan Instruments Inc.) by gently gripping their

tails, positioning them away from the direction of rotation. To maintain balance, the mice had to walk forward on the rod. The rota rod was set at a height of 30 cm above the ground and featured a rotating rod with a 3 cm diameter. The duration each mouse managed to stay on the rod for 180 s was recorded, excluding falls occurring within the initial 5 s due to improper placement by the experimenter [21]. A soft padded surface was positioned at the base of the apparatus to cushion any falls.

Alcohol preference test

For preference testing, individually housed mice (N = 10 mice per group) were used. Throughout a 24 h period, the mice had access to two conical tubes with a drinking spout attached filled with water. In order to institute a baseline, both tubes were initially filled with 40 mL of water and placed above the cages for three consecutive days. During the preference measurement phase, one of the tubes was replaced with a solution containing 8% alcohol. The amount of alcohol consumed by each animal was recorded over five consecutive days between 10 and 11 AM. To ensure unbiased positioning, the placement of the tubes within the various cages was randomized with regard to the side of the cages they were placed on. In all experiments, the ratio of alcohol to water consumed, and the total fluid consumption, were calculated to obtain a preference ratio. Additionally, half of the animals in each group (N = 5) were injected with WIN daily for five consecutive days. The alcohol preference ratio was determined by dividing the amount of alcohol consumed by the total fluid (alcohol + water) consumption [21].

Cytokine assay

Mice involved in the acute behavioral experiments were continuously administered either the vehicle or alcohol for seven consecutive days. On the eighth day, the mice were decapitated, and their brains were removed from the skull. To aid dissection, the brains were promptly frozen in liquid nitrogen. Specific brain regions containing the hippocampus were dissected and placed in cell lysis buffer. Using an ultrasonic homogenizer, the tissue was homogenized. The resulting homogenates were then centrifuged at 10,000 RPM for 5 min to separate the tissue debris. Samples of the resulting supernatants were collected and, after determining the protein concentration, frozen and stored at −80°C until needed for cytokine analysis. To profile the expression of IL-1α (interleukin-1α), IL-1β (interleukin-1β), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor-α), a Mouse Inflammation ELISA Strip kit (Signosis, Sunnyvale CA, USA) was employed. In brief, 100 µL of the diluted cell lysate sample was added to wells coated with a specific primary antibody against each cytokine. After incubation for 1 h at room temperature, the wells were aspirated and washed three times with 200 µL of assay wash buffer. Subsequently, 100 µL of a biotin-labeled antibody mixture was added to each well and incubated for 1 h at room temperature. The wells were again aspirated and washed three times with 200 µL of assay wash buffer. Then, 100 µL of streptavidin-HRP conjugate was added to each well and incubated for 45 min at room temperature. Following aspiration and another round of washes, 100 µL of substrate was added and incubated for 10 min, followed by the addition of 50 µL of stop solution to each well. The optical density of each well was measured using a microplate reader at 450 nm [21].

Statistical analysis

Data are presented as mean ± SEM. Sigma Plot 12.0 statistical program was used. Prior to performing the tests, we conducted a normality test (Shapiro-Wilk) to verify the distribution of the data. The statistical analysis was performed by the two-way analysis of variance (ANOVA). Post hoc comparisons of means were carried out with Tukey's test for multiple comparisons when appropriate. We used two-way ANOVA for the analysis of behavioral and cytokine assay data. Data from the alcohol preference study were analyzed by using repeated measures two-way ANOVA. The confidence limit of $p < 0.05$ was considered statistically significant. One of the factors of the ANOVA was the genotype (DAT-*Cnr2*, Cx3Cr1-*Cnr2* or WT mice) and the other factor was treatment groups (vehicle or alcohol).

Results

Brain CB2Rs modifies locomotor activity induced by alcohol

We evaluated acute motor activity in C57, DAT-*Cnr2*, and Cx3Cr1-*Cnr2* mice following the administration of 8% alcohol using an activity monitor apparatus. The results showed significant main effects for both treatment and genotype ($F_{1, 30} = 70.30$, $p < 0.001$ and $F_{2, 30} = 81.53$, $p < 0.001$, respectively), as well as a significant interaction between treatment and genotype ($F_{2, 30} = 16.22$, $p < 0.001$). Post-hoc analysis using Tukey's test for multiple comparisons revealed that alcohol administration significantly increased the total distance traveled in the activity box compared to the control group treated with vehicle. Interestingly, the results also indicated that specific deletion of CB2R in dopamine neurons (DAT-*Cnr2* cKO) enhanced alcohol-induced locomotor activity, with a statistically significant ($p < 0.01$) increase in the total distance traveled compared to WT mice. In contrast, the cell-type specific deletion of CB2R in microglia (Cx3Cr1-*Cnr2*

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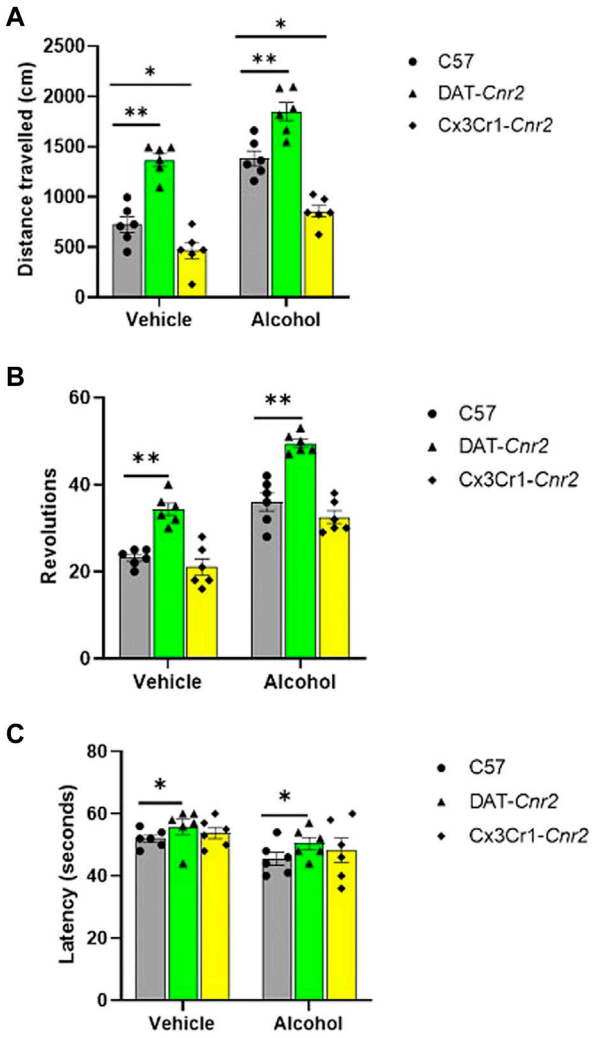

FIGURE 1
Acute effect of alcohol (0.8 g/kg) on distance travelled (centimeters) in the activity monitor apparatus **(A)**, on the absolute number of revolutions in the wheel-running test **(B)** and on fall latency (seconds) in the rotarod test **(C)** in WT, DAT-*Cnr2*, Cx3Cr1-*Cnr2* mice. Values are mean ± SEM (n = 6 in each group). Statistical analysis was done using Two-way ANOVA test. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to C57-WT group.

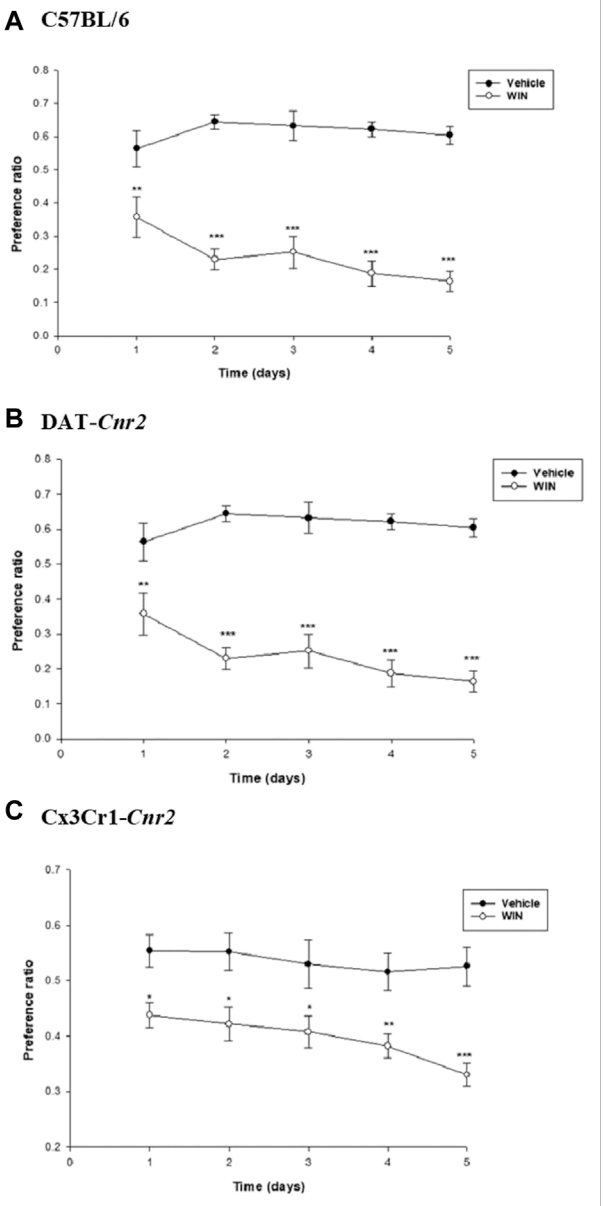

FIGURE 2
Role of WIN in alcohol preference. WIN significantly reduced alcohol preference in C57 wild type **(A)**, DAT-*Cnr2* **(B)** and Cx3Cr1-*Cnr2* **(C)** mice compared to vehicle treated controls. Values are mean ± SEM (n = 6 in each group). Statistical analysis was done using Repeated Measures Two-way ANOVA test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

cKO) reduced alcohol-induced locomotor activity, showing a statistically significant ($p < 0.05$) decrease in the total distance traveled compared to WT mice (Figure 1A).

Dopamine and microglia specific deletions of CB2Rs enhance alcohol induced wheel-running activity

In this study, we investigated acute wheel running behavior in C57, DAT-*Cnr2*, and Cx3Cr1-*Cnr2* mice following the administration of 8% alcohol using a mechanical wheel running apparatus. The number of revolutions exhibited a significant association with the treatment groups ($F_{1,\ 30}$ = 112.2, $p < 0.001$) and genotype ($F_{2,\ 30}$ = 56.12, $p < 0.001$). Post-hoc analysis using Tukey's test revealed that both the vehicle and alcohol treatment of DAT-*Cnr2* mice resulted in a significant ($p < 0.01$) increase in the absolute number of

revolutions compared to the control group of WT mice (Figure 1B).

Deletion of CB2R in dopamine neurons enhances alcohol induced reduction in fall latency

We examined the ability of mice to maintain their position on a rotating cylinder following the administration of 8% alcohol. We employed a constant speed rotarod apparatus for this assessment in C57, DAT-*Cnr2*, and Cx3Cr1-*Cnr2* mice. The results showed that cell type specific deletion of CB2R in dopamine neurons enhanced alcohol induced reduction in fall latency in the rotarod test of DAT-*Cnr2* mice, whereas this effect was not observed in the deletion of CB2R in microglia of Cx3Cr1-*Cnr2* mice. There was a significant main effect for both treatment and genotype ($F_{1,\ 30} = 28.43$, $p < 0.001$ and $F_{1,\ 30} = 62.52$, $p < 0.001$, respectively). Post-hoc analysis using Tukey's test indicated a statistically significant ($p < 0.05$) increase in fall latency in DAT-*Cnr2* mice compared to the WT controls. However, the cell-type specific deletion of CB2R in microglia did not affect the alcohol-induced changes in fall latency when compared to the WT controls (Figure 1C).

WIN 55,212-2 reduces alcohol preference in the wild type and cell-type specific CB2R cKO mice

We further investigated the potential association between subacute treatment with WIN and alcohol preference. In WT mice, the results demonstrated a significant effect of both treatment and time on the alcohol preference ratio ($F_{1,\ 20} = 79.229$, $p < 0.001$ and $F_{4,\ 20} = 3.172$, $p < 0.05$, respectively), as well as a significant interaction between treatment and time ($F_{4,\ 20} = 6.421$, $p < 0.05$). Post hoc analysis revealed a significant ($p < 0.01$) reduction in alcohol preference in mice treated with WIN compared to the vehicle-treated controls (Figure 2A). Similarly, in DAT-*Cnr2* mice, there was a significant effect of both treatment and time on the alcohol preference ratio ($F_{1,\ 20} = 233.855$, $p < 0.001$ and $F_{4,\ 20} = 4.956$, $p < 0.05$, respectively), along with a significant interaction between treatment and time ($F_{4,\ 20} = 9.042$, $p < 0.001$). Post hoc analysis indicated a significant ($p < 0.01$) reduction in alcohol preference in mice treated with WIN compared to the vehicle-treated controls (Figure 2B). In Cx3Cr1-*Cnr2* mice, statistical analysis also revealed a significant effect of both treatment and time on the alcohol preference ratio ($F_{1,\ 20} = 68.225$, $p < 0.001$ and $F_{4,\ 20} = 5.716$, $p < 0.05$, respectively), as well as a significant interaction between treatment and time ($F_{4,\ 20} = 2.812$, $p < 0.05$). Post hoc analysis demonstrated a significant ($p < 0.05$) reduction in alcohol preference in mice treated with WIN compared to the vehicle-treated controls (Figure 2C).

CB2Rs reduce alcohol induced increase in pro-inflammatory cytokines in mice hippocampus

The result form the cytokine study showed both treatment and genotype significantly affected the expression of TNF-α [treatment effect: $F_{1,\ 30} = 29.33$, $p < 0.001$; genotype effect: $F_{2,\ 30} = 20.51$, $p < 0.001$; treatment X genotype interaction: $F_{2,\ 30} = 5.43$, $p < 0.05$] and IL-1β [treatment effect: $F_{1,\ 30} = 12.27$, $p < 0.001$; genotype effect: $F_{2,\ 30} = 16.43$, $p < 0.001$; treatment X genotype interaction: $F_{2,\ 30} = 9.62$, $p < 0.01$]. Compared to the WT controls, Tukey's test revealed that there was statistically significant increase in the levels of TNF-α and IL-β, as evidenced by enhanced absorbance values, in DAT-*Cnr2* and Cx3Cr1-*Cnr2* mice treated with alcohol (Figures 3A–D).

Discussion

Due to the neuro-immune functioning associated with the reward pathway, recently, there is an increasing interest and attention on CB2Rs as a target for the treatment of drug addiction [31–34]. The aim of this study was to examine the effect of genetic and pharmacological modulation, using the non-selective CBR agonist WIN 55, 212-2, of CB2Rs on behavior and neuro-immune changes induced by alcohol. The results demonstrate that cell-type specific deletion of CB2Rs in dopamine neurons and microglia significantly altered locomotor activity, and wheel running activity, and on the rota rod performance test. The results also revealed that cell-type specific deletion of CB2Rs enhanced alcohol-induced inflammation. In addition, pharmacologic activation of CB2Rs using WIN 55, 212-2 reduced alcohol preference.

The results of the current study support our earlier finding that CB2Rs acts as a "brake" on dopamine neurons' ability to activate the locomotor system and that its deletion in DAT-*Cnr2* cKO mice improves psychomotor behavior [21, 28, 35]. The observation that deletion of CB2Rs in DA neurons resulted in enhanced spontaneous motor activity reinforces the notion that CB2R mediates inhibition of spontaneous movement via modulation of the dopamine system, probably through reduction of neuronal firing frequency [36]. However, in contrast to the DAT-*Cnr2* mice, Cx3Cr1-*Cnr2* mice showed a reduction in locomotor activity compared to the wild type controls. *In vitro* and *in vivo* studies demonstrated that activation of CB2R decreases inflammation and protect neurons from degeneration [26, 27]. In this study, the hypolocomotion observed in the Cx3Cr1-*Cnr2* mice might be due to lack of the neuroprotective effects of CB2Rs from neurodegeneration.

Alcohol dose, route of administration, and mouse strain all have an impact on how alcohol affects locomotor activity in mice. In this work, we discovered that locomotor activity was increased in both the wild-type and genetically modified mice after sub-

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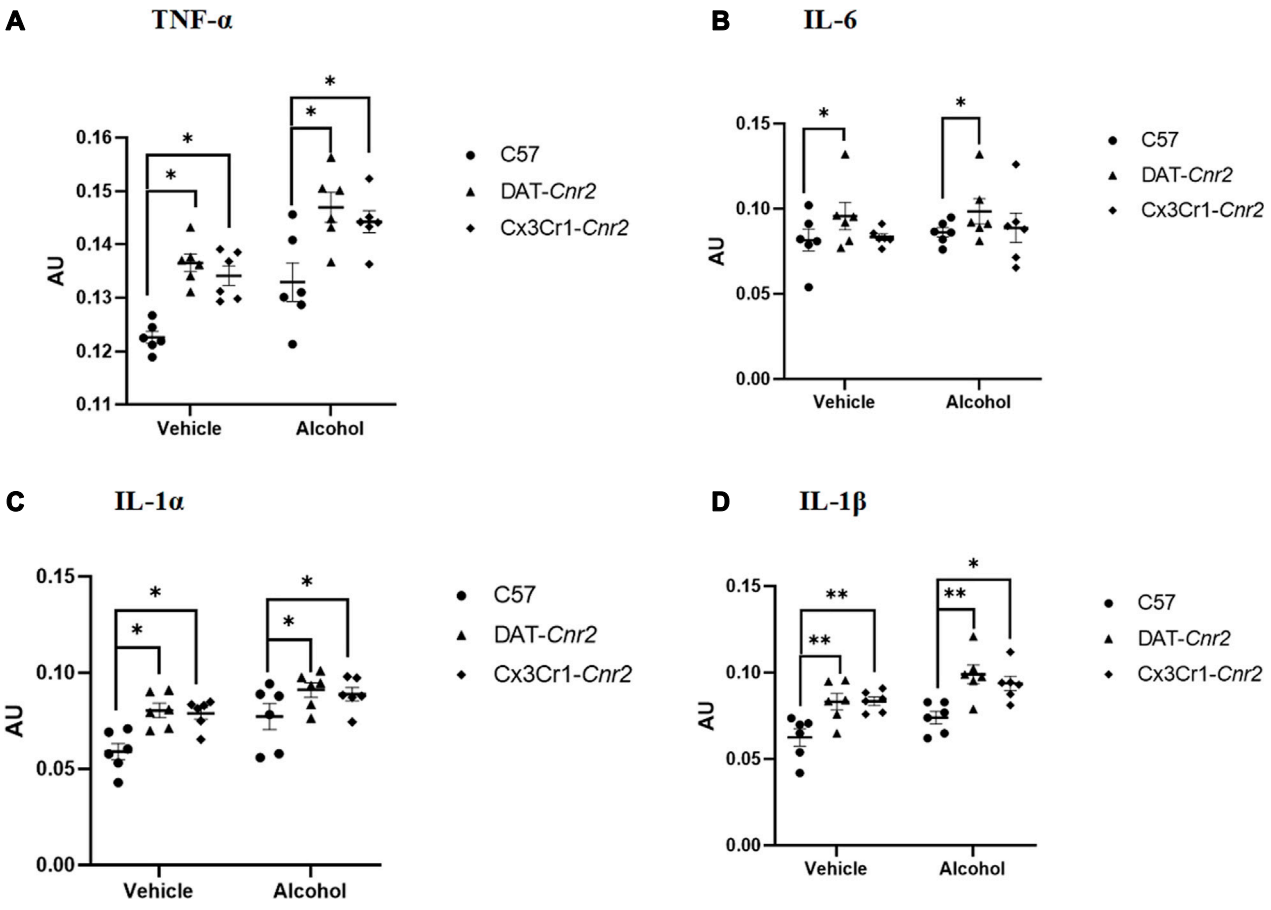

FIGURE 3
Measures of the levels of proinflammatory cytokines TNF-α **(A)**, IL-6 **(B)**, IL-1α **(C)** and IL-1β **(D)** in the hippocampus of mice (WT, DAT-*Cnr2*, Cx3Cr1-*Cnr2*) after seven consecutive days of sub-acute treatment with vehicle or alcohol (0.8 g/kg). Statistical analysis was done using Two-way ANOVA followed by Tukey's multiple comparisons test. Values are mean ± SEM (n = 4 in each group). $^{**}p < 0.01$, $^{*}p < 0.05$. AU – absorbance unit.

acute i.p. administration of 8% alcohol. Previous research have shown that alcohol enhances locomotor activity and locomotor sensitization [37–42], which is consistent with the findings of the present investigation.

Our investigation into the subacute effects of the WIN compound on alcohol preference revealed that it greatly decreased alcohol intake in DAT-*Cnr2* and Cx3Cr1-*Cnr2* cKO mice, providing one piece of support for the idea that CB2Rs are involved in the behavioral effects of alcohol. In our previous study we showed that the DAT-*Cnr2* cKO mice consumed less alcohol than wild type mice with and without the stress, suggesting that the deletion of CB2Rs in DA neurons contributed to the reduction in alcohol consumption and preference [28]. Studies showed contradicting result on the effect of CB2Rs on ethanol intake. Some reported that a naturally available full-agonist of CB2Rs, beta-caryophyllene (BCP) lowered ethanol intake in the two bottle paradigm in mutant *Cnr2*$^{-/-}$ mice [20, 43] whereas, others reported that sub-chronic injection of JWH015 enhanced alcohol intake in mice [44, 45]. The variation in response might be due to different factors such as concentration and route of administration of ethanol, duration of exposure, strain of animal and the animal model used in the experiment. However, accumulating data support a role of CB2Rs in modulating the addictive effects of alcohol indicating that CB2Rs might be targeted in the treatment of behavioral impairment induced by alcohol consumption.

Alcohol causes organ damage that affects the liver, cardiovascular system, and brain. This organ damage is characterized by inflammation and altered innate immune responses [46–48]. Chronic alcohol consumption results in neuroinflammation [49] and neurodegeneration in humans as well as animal models, as evidenced by increased expression of MCP-1, TNF-α, IL-1β and caspase-3 in the brain [48, 50, 51]. The hippocampus has been repeatedly affected by the neuroimmune dysregulations induced by alcohol [52]. Here we report that cell-type specific deletion of CB2Rs *per se* and administration of alcohol to CB2R cKO mice increased the expression of proinflammatory cytokines TNF-α, IL-6, IL-1α and IL-1β in the hippocampus of mice, which is an evidence for the neuroprotective role of CB2Rs. The use of CB2R ligands in the

neuroprotective and anti-inflammatory activity linked to neuropsychiatric and neurodegenerative disorders is based on the fact that CB2Rs expression is increased during injury and inflammation, with their upregulation during CNS disorders providing a basis and focus of attention [33, 53]. Studies showed that the activation of CB2R is related to decreases in pro-inflammatory cytokines (TNF-α, interferon gamma (IFN-ɣ), IL-1, IL-2, IL-6 or IL-12) [21, 54–56]. The outcome of our current investigation points to a critical role for CB2Rs and neuroinflammatory processes in alcohol-related neurobiological and behavioral changes. However, it should be noted that complete loss of the anti-inflammatory CX3CR1 receptor in homozygous mice is a potential confounder since this receptor is important for sustaining normal microglia function and lack of CX3CR1 reportedly results neurotoxic microglia phenotype. To prevent alcohol-induced neuroinflammation and related brain dysfunctions, pharmacological regulation of CB2Rs may be a focus. In summary, cell-type specific deletion of CB2Rs enhances psychomotor activity and increases the level of proinflammatory cytokines in the hippocampus. In addition, pharmacologic modification of CB2Rs using the WIN 55,212-2 compound reduced alcohol consumption in mice compared to vehicle. However, more studies are required to provide additional molecular and cellular mechanisms associated with neuro-immuno-eCB modulation of the effects of alcohol and CB2Rs in autoimmune disorders.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by William Paterson University IACUC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

The research was conceived and designed by BK and EO. The experiments and data collection were carried out by AR, MC, LD, NN, ME, AK, SE, JF, and BK. BK analyzed the data and prepared the initial draft of the manuscript. Q-RL provided technical support, and Q-RL and EO revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adolescent alcohol drinking interaction with the gut microbiome: implications for adult alcohol use disorder

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Reciprocal communication between the gut microbiota and the brain, commonly referred to as the "gut-brain-axis" is crucial in maintaining overall physiological homeostasis. Gut microbiota development and brain maturation (neuronal connectivity and plasticity) appear to be synchronized and to follow the same timeline during childhood (immature), adolescence (expansion) and adulthood (completion). It is important to note that the mesolimbic reward circuitry develops early on, whereas the maturation of the inhibitory frontal cortical neurons is delayed. This imbalance can lead to increased acquirement of reward-seeking and risk-taking behaviors during adolescence, and consequently eventuate in heightened risk for substance abuse. Thus, there is high initiation of alcohol drinking in early adolescence that significantly increases the risk of alcohol use disorder (AUD) in adulthood. The underlying causes for heightened AUD risk are not well understood. It is suggested that alcohol-associated gut microbiota impairment during adolescence plays a key role in AUD neurodevelopment in adulthood. Furthermore, alcohol-induced dysregulation of microglia, either directly or indirectly through interaction with gut microbiota, may be a critical neuroinflammatory pathway leading to neurodevelopmental impairments and AUD. In this review article, we highlight the influence of adolescent alcohol drinking on gut microbiota,

Abbreviations: ALD, alcoholic liver disease; AUD, Alcohol Use Disorder; AW, alcohol withdrawal; AD, Alzheimer's Disease; BBB, blood-brain-barrier; BAL, blood alcohol level; CDC, Centers for Disease Control; CNS, central nervous system; DA, dopamine; DR1, Dopamine Receptor D1; FASD, fetal alcohol syndrome disorder; FFAR, free fatty acid receptor; GABA; gamma amino butyric acid; GF, germ-free; GM, Gut microbiota; GBA, Gut-brain-axis; GPCR, G protein-coupled receptors; IL, interleukin; LPS, lipopolysaccharide; NFκB, nuclear factor-κB; NIAAA, National Institute of Alcohol Abuse and Alcoholism; NMDA, N-methyl-D-aspartate; PFCX, prefrontal cortex; SAMHSA, Substance Abuse and Mental Health Services; SCFA, short chain fatty acids; SPF, specific pathogen-free; TBI, traumatic brain injury; TLRs, Toll-like Receptors.

gut-brain axis and microglia, and eventual manifestation of AUD. Furthermore, novel therapeutic interventions via gut microbiota manipulations are discussed briefly.

KEYWORDS

alcohol use disorder, gut microbiome, adolescence, gut microbiota, toll like receptors

Introduction

Adolescence is a transformative period of human growth bridging developmental chasm between childhood and adulthood. Around the beginning of puberty, critical hormonal, physical, behavioral, and neurodevelopmental changes occur, which culminate through teenage years, and develop further during the mid-20's [1]. These transformations bring about necessary cognitive and social skills to enable once dependent teens to function as mature and near independent adults [1, 2]. However, some of the consequences of the adolescent neurodevelopmental changes such as impulsivity, risk-taking, sensation-seeking, and novelty-directed behaviors may continue into adulthood. In addition, early maturation of the reward and motivational circuits combined with the protraction of the inhibitory control circuitries, lead to an imbalance between motivational and cognitive-control systems during adolescence. This imbalance can enhance risk-taking behaviors, including substance abuse [2, 3]. Indeed, initiation of alcohol and drug use, often in high doses, are common occurrence during adolescence. Considering the profound neurodevelopmental changes during this time and the ensuing behavioral consequences, adolescence may be considered as a time of both resiliency and vulnerability.

Adolescents often begin consuming alcohol despite their greater susceptibility to its damaging effects [4, 5]. Initiation of alcohol drinking in early adolescence enhances the risk of alcohol use disorder (AUD) in adulthood [5–8]. AUD is a complex brain disorder characterized by an impaired ability to cease or moderate drinking behavior despite adverse effects. Although the exact cause of AUD remains elusive, neurodevelopmental changes, including microglia activity and inflammatory consequences during adolescence, play a pivotal role [9, 10]. As neuronal maturation and refinement peak during adolescence, the process of pruning, entailing removal of weak synaptic connectivity and enhancement of myelination continue into adulthood [11]. Thus, there is a reduction of gray matter and an increase in the white matter volume [12–14]. This is accompanied by enhanced connectivity, which allows faster speed and efficiency of information flow across relatively distant regions [15–17]. Two distinct and notable circuits in this regard, are the mesolimbic reward pathway and the prefrontal cortex inhibitory circuit (PFCX), both of which are critically involved in the complex social and cognitive processes [18, 19]. The mesolimbic dopamine (DA) circuit, however, as mentioned earlier, matures early in adolescence, whereas there is a delay in PFCX development, resulting in a vulnerable window.

In this review, we focus on the neurodevelopmental stages of adolescence, including role of key players such as gut microbiota and microglia and the influence of alcohol use on these parameters. Moreover, potential exploitation of such components for therapeutic purposes are elaborated on.

Adolescent neurodevelopment

The term "adolescent" describes a young person in the process of developing from a child into an adult [20]. Adolescent neurodevelopment is conserved throughout evolution and across species, signifying its crucial importance in acquiring necessary behavioral skills for transitioning into adulthood [1, 3]. These skills include attaining heightened reward sensitivity, acquiring peer-directed social interaction, and cognitive enhancement, all of which are essential in achieving maturity [2, 3, 21, 22]. Heightened reward sensitivity is considered a milestone necessary to facilitate approach toward novel stimuli and learning from new surroundings and social interactions [23]. However, risk-taking, novelty-seeking, and sensation-seeking behaviors, may predispose adolescents to alcohol and drug use [24, 25]. Curiously, these behaviors may also be manifested in animals [26, 27], suggesting that certain neurobehavioral characteristics of adolescence may have biological causes.

Neuronal refinement during development

Neuronal refinement and maturation continue throughout adolescence, as at birth and even during adolescence, there are more neurons (about 4–5 times) than in adulthood [28–30]. Approximately 50% of the synaptic connections in selective regions are lost due to synaptic pruning [31], which is believed to ensure establishment of appropriate connectivity [31, 32], reduction in energy use, and increase brain efficiency [33, 34]. This process is also affected by myelination that begins early in life, peaks through adolescence and continues into adulthood [11]. Thus, increase in myelination and decline in synaptic connection help refine brain connectivity into the adult form [35]. However, myelination can be impacted by neurotoxic agents such as alcohol, which can poise great danger to the maturing brain.

Some of the adolescent synaptic pruning appears to be experience dependent [35]. For example, heightened stress exposure and alcohol consumption during adolescence, can affect neurodevelopmental resiliency [36]. On the other hand, enriched environment during adolescence can induce a variety of beneficial changes in the expression of genes in critical brain areas such as in striatum, an area that plays a pivotal role in motor and motivational behaviors [37]. Myelination is also experience-dependent as it helps stabilize axonal pathways [38]. It is believed that myelination, in concert with synaptic pruning help with the "rewiring" of brain, particularly the prefrontal cortex (PFCX), which is critical for many adult-type behaviors including cognitive functions [39].

Reward and impulsivity during brain development

DA system, essential for detecting, responding to, learning from reward, cognitive control, decision making and motivation [40, 41], undergoes significant transformation during adolescence [42]. Specifically, there is a loss of up to 50% of DA (D1) receptors in some areas, a compromised clearance of which, results in a reduction in social play and social exploration [43]. However, in other areas, DA activity may increase two-to seven-fold during adolescence [44, 45]. Thus, the mesolimbic DA pathway, considered to be the reward circuitry, is maximally developed in adolescence [18, 46, 47], which corresponds to peak in reward-seeking in mid-adolescence (i.e., approximately 14–15 years) that gradually declines into adulthood [25, 48, 49]. On the other hand, PFCX DA system, considered to be critical in inhibitory control of risk taking, has a protracted maturation [18, 50, 51]. This protraction results in developmental immaturities in cognitive control, attentional regulation, and response inhibition of behaviors [2], and may contribute to the persistence of certain maladaptive behaviors such as alcohol and drug use in adolescence [52, 53].

Dysregulation of PFCX behavioral control systems is associated with impulsivity, which contributes to alcohol seeking and use during adolescence, particularly, in stressful and arousing situations [54, 55]. This impulsivity may even continue into adulthood binge drinking (aged 18–30) [56]. Animal models of AUD also show impulsivity and risky choice behavior if PFCX is dysregulated, suggesting biological basis for such behavior [57, 58]. Excess alcohol use, in turn, by damaging neuronal cells, could lead to dysregulation in PFCX, further exacerbating aberrant behaviors (impulsivity/drug seeking) which can lead to drug addiction. Therefore, adolescent alcohol consumption can be considered a risk factor in AUD development in adulthood [59, 60]. Thus, delaying the onset of alcohol drinking, during this period of vulnerability, can significantly reduce the risk of AUD [2].

Environmental and non-neuronal factors during development

Adolescent engagement in risky behaviors commonly occurs in social situations [61–63]. Shaping and refinement of the brain neuronal system during this period is also impacted by exposures to environmental factors. Microbiomes, discussed in detail below, have recently attracted considerable attention as an important influencer of the brain function and affected by environmental factors such as diet, chemicals, etc. Interestingly, it was suggested that early life antibiotic-induced microbiota disruption may have subtle but enduring effects on the brain function and social behaviors [64].

Microglia, also discussed below in detail, are non-neuronal cells that constitute only 10% of the total CNS cells [65]. Nonetheless, they perform important task of surveying the environment and responding to insult [66, 67]. Microglia are considered CNS phagocytes, which also undergo significant changes during adolescence [68]. These changes contribute to neurodevelopmental fine-tuning [69–71]. Such as increase in brain efficiency, and synaptic pruning throughout cortical and limbic structures [71–73]. Moreover, by influencing early myelin formation and removing aberrant myelin [74], myelination is optimized. Interestingly, microglia may also play a role in dopaminergic circuits refinement which, as discussed above, are critical in reward-seeking and social behavior [75].

Microbiome and neurodevelopment

The gut microbiome (GM) is an ecosystem of 100 trillion commensal microbes, complex in composition and abundance, that mainly colonize the gut [76, 77]. Although the terms microbiota and microbiome are often used interchangeably, microbiota refers to the actual microbes, whereas microbiome refers to the microbes and their genes. The colonization of the gut starts at neonatal period and continues throughout life. During infancy, the ecosystem is unstable, but GM develops into a highly diverse and robust community in adulthood [78]. It was thought that the collective genome of microbiota, the microbiome, encodes 100 times more genes than the human genome [79]. However, recent in-depth analyses suggest only a slightly higher number of microbiomes compared to the human genome [80–83]. GM is essential for the maintenance of the host's health including innate and adaptive immune system [80], food digestion, fermentation of otherwise indigestible carbohydrates and fibers, energy production, synthesis of several vitamins (e.g., vitamins K and B) and the metabolism of bile acids, sterols, and xenobiotics [81, 82]. GM can produce or release neurotransmitters, choline and its metabolites as well as short chain fatty acids (SCFAs). These products are secreted into the gut lumen, transported across the epithelial barrier, and carried to the effector organs including the brain, via the

bloodstream. The gut microbiome, due to its immense impact on human equilibrium, immune function, neurology, mental health, and aging process, is now commonly referred to as a new metabolic "organ" [80–83].

Maturation of GM is critical for neuronal maturation and brain development [83]. Many studies show GM maturation parallels the temporal course of brain development. Using several experimental approaches, including germ-free (GF) animals, and antibiotics, host microbiota's effect on CNS functions have been studied [84–86]. For example, some antibiotics such as minocycline have profound acute effect on the microbiota diversity and composition [87, 88]. Moreover, the fact that most critical development of host immunity occurs within the first few years of life, which coincides with the maturation of the GM, reinforces the notion that GM is also involved in immune system development [89, 90].

The synchronized communication between the CNS and GM via GBA is critical in shaping the neurodevelopment and influences brain's biology under homeostatic conditions [91, 92]. Some of these functions include regulation of the permeability of BBB [93–95], and glial functions [91]. GM's metabolic products SCFAs, vagus nerve, and microbe-associated molecular patterns (MAMPs) (such as Toll-like receptors (TRLs) are the mechanisms purported to facilitate communication between GM and CNS [96]. Most TLRs, a family of pattern-recognition receptors that enable the recognition of conserved structural motifs of wide array of pathogens that drive inflammation, are expressed in the CNS, mainly in glial cells [97]. SCFAs monitor and integrate gut functions with emotional and cognitive centers of the brain. SCFA also regulate peripheral intestinal functions, intestinal permeability, and immune activation [98]. Indeed, microglia from GF-mice display a range of abnormalities that are dependent on GM SCFA. A specific pathogen-free (SPF) mice constitutively lacking the SCFA receptor FFAR2 displayed a similar aberrant phenotype to GF animals [99], suggesting that GM metabolite, SCFAs, and microglia are involved in the bidirectional crosstalk between GM and the brain.

It is not surprising, therefore, that dysbiosis or disruption of intestinal microbiota homeostasis can lead to variety of diseases [100], including cardiovascular [86], inflammatory bowel disease [101], and type 1 and type 2 diabetes [102, 103]. Common also, are CNS disorders such as anxiety, depression and substance abuse [82, 104, 105]. Dysbiosis can be caused by environmental factors including diet [106], disruption of circadian rhythms [107], and alcohol consumption [107], where the latter is discussed in more detail below.

Microglia and neurodevelopment

Microglia, considered the immune cells of CNS, are primarily responsible for neuroimmune responses and neuronal development [108, 109]. They facilitate the maturation and survival of neuronal progenitors and proper network integration during CNS development [110]. In general, there are three phases: early, pre- and adult microglia. Microglial maturation phases are defined by expression of a subset of genes corresponding to the core set of microglia functions [111]. Therefore, microglia show heterogeneous transcriptional profiles in the embryonic, early postnatal, and adult, depending on their microenvironment in CNS [112–114]. Early on, before BBB development, microglia derive from immature erythromyeloid progenitors, and migrate from the yolk sack blood islands to CNS [111, 115]. During late gestation and early postnatal development, embryonic microglia proliferate and colonize the whole CNS [111]. A few weeks after birth, microglia transition to "adult microglia" stage, in which they constantly survey their immediate surroundings and actively maintain homeostatic conditions by phagocytizing neuronal debris [116], and interacting with neighboring CNS cells [117]. They achieve these through the dynamic extension and retraction of their processes [118, 119].

Microglia can assume different phenotypes and retain the capability to shift functions to maintain tissue homeostasis depending on the influence of stimuli from the environment [120]. For example, during infection or injury, microglia switch from a homeostatic surveillance state to an activated state to facilitate antimicrobial or tissue repair to restore homeostasis [108]. Importantly, microglia can either be stimulated by GM toxin lipopolysaccharide (LPS) to a pro-inflammatory (M1) phenotype where they would express pro-inflammatory cytokines, or by IL-4/IL-13 to an anti-inflammatory (M2) phenotype for resolution of inflammation and tissue repair [120]. Given their dual role in immune and developmental functions, it may be expected that microglial dysregulation would contribute to neurodevelopmental disorders. Indeed, microglia overactivation could lead to neuronal damage and onset/progression of several neurodegenerative and neurodevelopmental disorders [121]. In addition to pro-inflammatory cytokines, other bioactive substances released from overactivated microglia, such as ROS and glutamate could also play a role in microglia-dependent neuroinflammation [122], and/or neuronal loss [123, 124].

Since microglia can also shape neurodevelopmental fine-tuning and complex neurodevelopmental programing [125], their transient reduction at critical stages of development can alters synaptic plasticity [126]. Microglia interaction with various cellular components including neuronal activity and synaptic formation, leads to establishment of novel functional neural network. Thus, microglia by inducing synapse formation during development, monitor the functional state of synapses in adulthood [70]. Moreover, during early brain development, microglia's main functions include synaptic remodeling, regulating the number of neurons through mechanisms of programmed cell death (apoptosis) [71, 125], and shaping of

the neuronal circuitry. Thus, early on in life, the brain being highly plastic, contains excess number of immature synaptic connections and is shaped by sensory experience [70]. The over-crowding of neurons is subsequently "pruned," or eliminated, primarily via microglia, to allow functional connectivity during development [126, 127]. In addition, microglia's involvement in myelination (via oligodendrogenesis), during development and throughout life, allows efficient and critical neuronal communication [74]. Curiously, recent evidence implicates microglia's own programmed cell death via pyroptosis, autophagy, and ferroptosis in neurodegenerative diseases, including Alzheimer's disease (AD) [128].

Microglial activity, governed in part by cytokines, chemokines, neurotransmitters, and other signaling molecules [129], is highly sensitive to environmental cues. As such, GM has emerged as a central player in microglial maturation and activation [120]. Sophisticated crosstalk between the CNS and the gut microbiome, and critical interdependency between microglia and GM, where the latter facilitates microglia's development, are now well-established [130]. However, the exact mechanisms of such communications are not well understood. Below, our current knowledge of GM-microglia interactions in relation to brain maturity and adolescent drinking are reviewed.

Microbiota-microglia interaction and neurodevelopment

That GBA plays a pivotal role in regulating microglial maturation and function during critical windows of development is well recognized [131, 132]. Microglia, in turn, is one of the key cellular intermediates linking CNS with GM. Distinct developmental stages are present during which there is heightened microglia susceptibility to immune mediators and environmental cues [110, 113]. For example, environmental exposure to chemicals such as alcohol can disrupt microglia development and maturation, primarily due to dramatic changes in microbiota. Similarly, antibiotics-induced loss of GM causes microglia to assume an immature status reminiscent of developing juvenile microglia [133]. On the other hand, recolonization of the gut with complex microbiota restores its plasticity, a finding that was also confirmed in mice born from GF maternal mice [134]. Indeed, GF-mice exhibit a wide range of microglia abnormalities including increased density and distribution across various brain regions and altered cytometric expression patterns for developmentally regulated proteins [99]. Moreover, such microglia are less reactive when challenged with LPS, again, suggesting GM's crucial role in microglia maturation and neuronal function [99]. Interestingly, microglial changes appear to be dependent on SCFAs, as specific pathogen-free (SPF) mice constitutively lacking the SCFAs receptor FFAR2 display a similar aberrant phenotype as seen in GF animals [99]. Furthermore, GF and antibiotic treatment not only disrupt typical microglial spatial network throughout the brain but also result in forming atypical contacts between processes of adjacent cells [99].

Importantly, however, is the finding that even transient perturbations in microglial function could have life-long effects on neuronal patterning, functional connectivity and behavior [135, 136]. Thus, it has been demonstrated that a transient reduction in microglia number at critical stages of development alters synaptic plasticity including differentiation and maturation of precursors into neurons or neurogenesis [126, 137]. This is because most newborn neurons undergo apoptosis and are phagocytosed by microglia as part of normal neurodevelopment. However, over time, this process becomes limited to neurogenic niches of the adult brain [127]. In addition, microglia not only play a critical role in debris clearance but may also facilitate neuroblast differentiation in response to signals [138]. Maternal immune activation results in accelerated microglial maturation that exhibit adult microglia phenotype [110, 113] and can present with detrimental consequences including neurological disorders that continue long after the microglia phenotype is restored [113]. On the other hand, microglia's expression of pro-inflammatory cytokines such as TNF- α, IL-1 β, and IL-6 and trophic factors, help mediate the interactions between the host's microbiome and the developing brain [139], resulting in refinement of functional neuronal circuits [131, 132, 140]. It was demonstrated recently that microglia's secreted factors directly increase differentiation of human neural stem cells to a dopaminergic lineage [83]. However, whether microglia are involved in heightened reward-seeking and/or risk-taking including development of AUD, remains to be determined.

Collectively, these findings suggest that bidirectional crosstalk between the gut and the brain may influence disease pathogenesis. Thus, alteration in GM during the early stages of development may have long-lasting effects on the GM composition throughout the lifespan with clear implications for the immune system as well as neuronal development. Because excessive alcohol consumption results in dysbiosis and microglia alteration, it is not surprising that AUD would be associated with neurological diseases [141]. Below, further association between alcohol, GM, and microglia in relation to neurodegenerative/neuropsychiatric disorders is elaborated.

Adolescent alcohol drinking

Alcohol is the most used drug among adolescents [142–144], where experimentation and initiation usually begins in early adolescence (50%–70% of 15 year-old use alcohol) [145], and peaks during young adulthood (18–24 years of age), where binge drinking is more common [146]. US prevalence of binge drinking in adolescents aged 12–17 and 18–25 are 4.7% and 34.9%

respectively [147]. Binge drinking is considered consuming 4 or more drinks for females and 5 or more drinks for males within 2 h [143, 148]. A single drink consists of about 14 g of pure alcohol, which is found in 12 ounces (355 mL) of regular beer (usually containing 5% alcohol); 5 ounces of wine (usually containing about 12% alcohol); or 1.5 ounces of distilled spirits, which is about 40% alcohol. Although, binge drinkers drink less frequently, they drink more alcohol per drinking episode achieving a blood alcohol level (BAL) topping 0.08% (>80 mg/dL) and hence increasing alcohol-associated risks and consequences [143, 148, 149]. A small percentage (10%) of binge drinkers are considered heavy binge drinkers, where 10 or more drinks are consumed per occasion, and yet 5% are extreme binge drinkers where over 15 drinks is consumed in a binge session [142, 150]. Epidemiological report indicates that early initiation of alcohol drinking before the age of 15 years increases the risk of AUD in adulthood by fourfold [6, 7]. About 30%–40% of adolescent binge drinkers, i.e., 1.6% of 12–17 year-olds and ~14% of older adolescents, meet criteria for AUD [147, 151, 152]. Although males are overrepresented in the extreme binge drinkers, the gender gap is narrowing [142, 153].

In addition to adolescent drinking, individuals with fetal alcohol syndrome disorder (FASD), a heterogeneous group of conditions defined as the physical, behavioral, and learning impairments that occur in the offspring of women who drank alcohol during pregnancy, may also exhibit increased risk of substance abuse including AUD in adulthood. Thus, alcohol exposure may impact behavioral outcomes throughout neurodevelopmental period where the earlier the exposure, the worse the outcome [154]. However, disentangling underling factors in each case remains a challenge [155].

It is noteworthy that adolescents, compared to adults, are insensitive to various intoxicating effects of alcohol such as motor incoordination, social impairment, and sedation [3]. It is thought that adolescent-typical insensitivities to aversive stimuli in the presence of greater reward sensitivity contribute to their proclivity to associate more benefit and less cost to alcohol and drug use. This could encourage pursuit of or continued engagement in risky activities, particularly when prior activities proved rewarding but without disastrous consequences [62, 63, 156].

Alcohol use disorder and microbiome

A potential connection between GM and AUD was suspected since mid 1980s. Initially, the role of GM in alcoholic liver disease was intensely investigated. Later, possible role of GM in addiction to alcohol was advocated. With our advancement in understanding of the GBA, it is anticipated that novel GM-targeted therapies will become available [157].

It is important to reiterate that harmful consumption of alcohol (alcoholism) is responsible for approximately 5.3% annual deaths in all age groups, and at an alarming rate of 13.5% for the younger age group of 20–39 years old [144]. Although alcoholism has been studied for decades, only relatively recently the examination of gastrointestinal (GI) microbiome and its impact on AUD has been intensely investigated. An initial observation reported that the content of Gram-negative anaerobic bacteria in jejunal aspirates from alcoholic individuals were significantly higher compared to control individuals [158]. Animal studies, confirmed this involvement where it was shown that more than 10 weeks of ethanol ingestion in rats led to significant dysbiosis of the colonic microbiome [159]. In subsequent years, many sequencing studies of the microbiome from rodent models of alcoholism, humans with AUD, as well as non-human primate studies of addiction have solidified GBA's importance in alcohol addiction [157].

Thus, GM not only plays an important role in development of AUD but also in a variety of neurological and neuropsychiatric diseases including Parkinson's disease, Alzheimer's disease, depression, and autism spectrum disorder [160–162]. Chronic alcohol consumption can cause changes in the composition of GM and impair the gut mucosal barrier as well as homeostasis. Once the mucosal barrier is compromised, LPS from GM is released and translocated to peripheral blood circulation, where it acts on TLR4 [163]. Activation of TLR4 can lead to increases in proinflammatory cytokines which further disrupt BBB and hence result in further neuroinflammation [164], a major contributor to AUD. For these reasons it has been suggested that the gut–brain axis might be a potential target to reduce alcoholic relapse risk.

In addition to the central effects of AUD, GM dysbiosis, can lead to liver disease. Indeed, GM changes occur in parallel to liver injury, with an increase in endotoxin-producing bacterial taxa, leading to cirrhosis and alcoholic hepatitis. In this regard, AUD effect on GBA can further potentiate alcohol misuse and hasten hepatic encephalopathy. Thus, strategies that address both alcohol cessation and microbiota alteration are needed for meaningful improvement in all AUD spectrum [165].

Furthermore, a plethora of indirect evidence point at the involvement of GM dysbiosis in microglia activation (discussed below) and AUD. For example, GM metabolite SCFAs can cross BBB and affect microglia directly [166]. Both infiltrating macrophages and microglia become activated in response to tissue damage and can release proinflammatory cytokines, which may contribute to neuroinflammation and BBB breakdown [167, 168]. Orally administrated mixture of the three major SCFAs acetate, propionate and butyrate can sufficiently drive maturation of microglia [169]. Of these, butyrate has been demonstrated to possess multiple benefits, including enhancing the gut barrier, reshaping the gut microenvironment, and repressing the inflammatory progression. Moreover, butyrate has shown to be neuroprotective against alcohol toxicity in an *in-vitro* model [170].

Alcohol abuse via changes in GM composition and metabolic function can lead to oxidative stress and leaky gut (allowing

bacterial passage into the lumina), and subsequent development of alcohol-related diseases [81, 171]. Also, GM dysbiosis by disrupting microglial maturation and activation can causes behavioral changes associated with AUD. However, despite frequent reports of dysbiosis in AUD patients, microbiome-targeting therapies for this disorder awaits clinical trials (see also below for more detail).

Alcohol use disorder and microglia, and role of toll-like receptors

Microglia involvement in AUD pathology is amply supported by the findings that prolonged and heavy exposure to alcohol can not only lead to appreciable reduction in glial cell numbers in both temporal and frontal cortices [172], but also to impairment of neuronal and glial cell functionality [173]. In the developing brain, these effects are more pronounced and extend to cerebral white matter, corticolimbic system and cerebellum (especially the vermis) [173]. Cortical microglia, however, show remarkable morphological plasticity as they rapidly deactivate following acute severe alcohol exposure [174]. Following chronic high alcohol exposure, there is a marked increase in microglia activation [167, 175], accompanied by high levels of proinflammatory mediators and reactive oxygen species that can lead to tissue damage and cell death [103]. Conversely, chemical depletion of microglia, can block the production of inflammatory mediators in the brains of mice after acute binge ethanol withdrawal [176].

Epidemiological studies, based on FASD, also suggest a role for microglia in early neurodevelopment [177], as areas that are dependent on neuroglial cells for their formation such as corpus callosum and anterior commissure exhibit abnormal glial migration [178] and underdevelopment [179]. Moreover, during brain growth spurt, characterized by rapid glial cell proliferation and maturation, ethanol exposure can lead to microencephaly, suggesting potential effect of ethanol on proliferation, growth, and maturation of glia [180]. Likewise, during adolescence, binge drinking causes devastating effects as reflected in morphological changes in hippocampal microglia that can last over 1 month [181]. Accompanied neuroinflammatory processes induce behavioral changes such as sedation and alcohol withdrawal symptoms including memory impairment, neuronal cell death and diminished neurogenesis [182, 183]. Insensitivity to sedative effects to alcohol, blackouts and kindling, contribute to exacerbation of withdrawal episodes with each cycle of withdrawal during adolescence [184, 185].

Chronic alcohol consumption induces microglia proliferation [167, 186, 187] and microglia morphological changes reflective of a proinflammatory phenotype in a context-dependent manner [9, 186]. During context-dependent activation of microglia, prior insults are recalled, resulting in amplified responses to a second inflammatory insult [188, 189]. This suggests that prior ethanol exposure potentiates a subsequent microglia response that is primed by initial alcohol exposure. Alcohol can directly activate microglia to increase expression of proinflammatory chemokines and cytokines. The chemokines and cytokines in return, can alter sensitivity to alcohol-induced sedation, alcohol withdrawal severity [182], memory impairment [183], as well as alcohol drinking patterns [190].

Alcohol-enhanced microglia-specific immune responses can be blocked by minocycline, a microglia activation inhibitor [191]. This blockade of microglia immune response alters alcohol-induced motor impairment decreases alcohol self-administration in mice [192], and attenuates withdrawal-induced anxiety and relapse drinking in rats, suggesting that microglia may be the critical mediator of alcohol behavioral effects [193]. Minocycline also reduces traumatic brain injury (TBI) induced by microglial activation [194]. Since alcohol use is associated with microglial activation, it would be reasonable to expect that adolescent binge drinking may enhance TBI. However, the effects of adolescent binge drinking on microglia and potential use of minocycline in AUD remains to be investigated.

Adolescent alcohol drinking impacts central inflammatory cells and signaling molecules [167]. Sensitized microglia can interfere with homeostasis by decreasing expression of homeostatic genes [195]. For example, several genes in Toll-like receptor (TLR) signaling pathways are activated by alcohol [96]. TLRs are important mediators of inflammatory pathways in the gut and play a crucial role in maintaining the balance between commensal bacteria in the gut and the mucosal immune system [196]. TLRs are evolutionarily conserved receptors belonging to the family of pattern recognition receptors (PRRs) which play a vital role in immune responses. Indeed, TLRs hold a key position in the first line of defense against pathogens because of their ability to recognize the conserved pathogen-associated molecular patterns (PAMPs) that are conserved structures of the pathogens. Activation of PRRs results in the downstream transcriptional activation and expression of numerous inflammatory mediators. In addition, PRR signaling also leads to the triggering of various processes involved in autophagy, cell death, cytokine processing, and phagocytosis. Thus, TLRs are directly involved in the regulation of inflammatory reactions and activation of the innate or adaptive immune responses for the elimination of infectious pathogens and cancer debris [196].

To date, 222 TLRs have been identified in invertebrates and 28 TLRs in vertebrates. Depending upon their functionality and location in the host cell, TLRs are further categorized into two types: 1. Cell membrane TLRs, which are expressed in their active form on the cellular surface. They include TLR1, 2, 4, 5, 6, and 10.2. Intracellular TLRs, which are expressed within the host cells on the organelle biomembranes like endoplasmic reticulum (ER), endosomes, and lysosomes. They include TLR3, 7, 8, and 9 [196]. TLR4 is the major pattern recognition receptor of bacterial

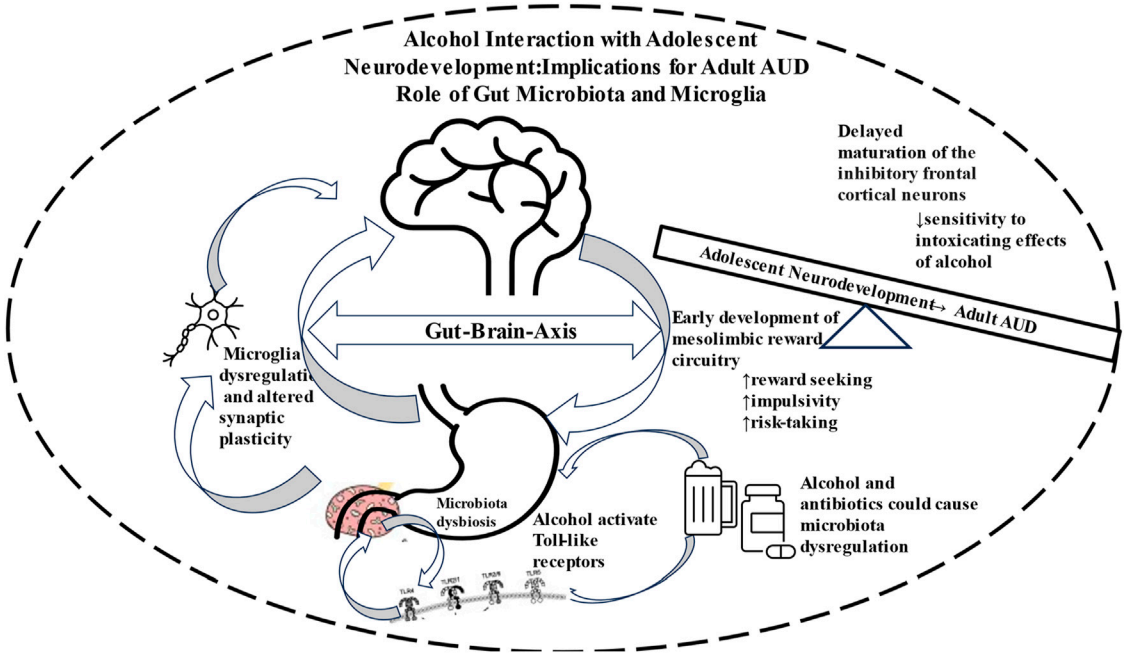

FIGURE 1
Schematic diagram depicting involvement of Gut-Brain Axis in neurodevelopment that renders the adolescents more vulnerable to drug seeking behavior and eventual manifestation of alcohol use disorder (AUD).

endotoxin, LPS [163]. Although endotoxins are not generally believed to cross BBB [197], they can induce proinflammatory microglia. Indeed, in TLR4 knockout and postmortem tissue of AUD patients, there is breakdown of BBB [198]. Interestingly most of the TLRs are expressed in microglia and astrocytes [164, 199, 200]. n addition to microglia, peripheral macrophages can be recruited into the CNS under pathologic conditions and may serve to amplify ongoing neuroinflammation [201]. Alcohol's activation of TLRs triggers downstream stimulation of nuclear factor-κB (NFκB) and the induction of genes that encode inflammation-associated molecules such as cytokines [202, 203]. Thus, activation of the TLRs can significantly contribute to neuroinflammation [204]. Indeed, increased TLR4 activation is often the reason for neurodegeneration exacerbation [205]. Hence, it may be concluded that at least some of neurodegenerative consequences of heavy alcohol drinking might be mediated via TLR4 stimulation.

As mentioned earlier, adolescent exposure to alcohol significantly increases the risk of AUD in adulthood. Although the reason(s) behind this association is not fully known [206], it may be speculated that alcohol's priming effect of microglia or changes in TLRs may have major roles. Interestingly, TRLs are also involved in bidirectional communication between GM and CNS and are believed to play an essential role in regulating intestinal barrier permeability and maintaining intestinal microbial homeostasis. The intestinal microbiota, in turn, plays an essential role in TLR ligand activation and distribution [207]. Thus, alcohol-induced dysbiosis in adolescence may be a major contributory factor to AUD development in adulthood. This discovery, as discussed below, may present with novel interventions in AUD.

Possible microbiome directed therapies against alcohol use disorder

Based on above discussion, it is likely that manipulations of GM may offer a novel intervention in AUD. In this regards, fecal microbiota transplantation (FMT) in patients with alcoholic liver disease [208, 209]. and more recently for the treatment of AUD in general, has been attempted [210]. The latter study noted a reduction of serum IL-6, reductions in craving, cognitive functioning improvements, and reduction in negative psychosocial impacts following administration of *Lachnospiraceae* and *Ruminococcaceae*. The authors also reported an increased abundance of *Roseburia* in FMT-recipients. Interestingly, *Faecalibacterium* and *Roseburia* have been implicated to have a protective role on GBA and intestinal epithelium in alcoholism [211, 212]. Thus, the possibility exists that by restoration of beneficial bacteria significant improvement in CNS health can be achieved. Moreover, manipulation of TLRs as discussed above, could offer additional targets. It is anticipated

that with continuous studies in this field, further refinement of treatment modalities involving GM in addiction in general and AUD, in particular may be achieved [207].

Other therapeutic potentials

In addition to manipulation of GM, extensive effort is being expended in understanding the neurobiological substrates of AUD with the hope of discovering effective novel targets [212]. As it currently stands, three approved medications are available to combat alcoholism or AUD, aiming to stop or reduce the drinking habit and prevent relapse. These include disulfiram, an inhibitor of the degrading enzyme aldehyde dehydrogenase, that acts by inducing aversion nalmefene or naltrexone, antagonists of opioid receptors that act by blunting the rewarding effects of alcohol, and acamprosate, a gamma amino butyric acid (GABA) synthetic analog that acts by modulating or antagonizing NMDA receptors. The latter is primarily used for maintenance of abstinence from alcohol in detoxified alcohol-dependent patients [213]. However, all these medications are only modestly effective. In addition, about one in six people globally, is estimated to receive treatment, with the rate being at even lower in low and lower-middle-income countries [214]. For potentially life-threatening condition, manifested during withdrawal and believed to be caused by glutamate overactivity, benzodiazepine are the primary medications applied [215]. In addition, "talk therapy" or behavioral interventions, consisting of therapies that build motivation and teach skills for coping and preventing relapse, when combined with medications yield a better outcome. Physical activity may also be used as adjunctive treatment for severe AUD [216]. Potential application of neurosteroids, polyphenols, neuropeptides, modulators of nicotinic acetylcholine receptors [217], muscarinic acetylcholine receptors, glutamate receptors, GABA receptors, cannabinoid receptors, G protein-coupled receptors (GPCRs), tyrosine-kinase receptors as well as various nutrients such as carnitine, folic acid, selenium, omega 3 fatty acids and zinc were recently reviewed [212].

Discussion

Adolescence is a period of human development that span between childhood and adulthood. The neurodevelopmental transformations during adolescence are geared towards acquiring cognitive and social skills that are required to enable the dependent teen to eventually transform to an independent adult. However, some developmental or maturation imbalance in circuitries that control reward vs. inhibition in adolescence, can lead to increased presentation of risk-taking and reward-seeking behaviors, which can include heightened risk of substance abuse such as alcohol drinking. Mirroring the adolescent neurodevelopmental changes, the gut microbiota also undergoes significant maturation, and at the same time establishes a strong bidirectional communication with the brain. This reciprocal communication, referred to as GBA plays a crucial role in driving the behavioral changes associated with AUD.

There are emerging mechanisms by which altered microglial functions could contribute to several major etiological factors of AUD. Pre- and postnatal exposure to alcohol can modulate microglial cell phenotype and function, supporting the notion that reciprocal interactions between microglia and intestinal microbes could play a crucial role in AUD etiology. Alcohol-associated inflammatory signaling contributes not only to CNS inflammation and neurodegeneration but also to alcohol addiction.

Chronic and high alcohol use can cause GM dysbiosis, leading to neuroinflammatory condition via microglia activation and eventual manifestation of AUD (Figure 1). It is estimated that adolescents who begin drinking alcohol between the ages of 11–14 are 4 times more likely to develop AUD compared to peers that postponed drinking until after the age of 20.

Based on crucial role of GM and microglia in AUD manifestation, particularly during adolescence, and our deeper understanding of the interaction between these two systems, novel promising interventions are presented. However, further investigation on not only the efficacy of the approaches but also the potential role of gender and/or ethnicity in AUD manifestation and treatment are of crucial importance.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alcohol use and the pain system

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The World Health Organization's epidemiological data from 2016 revealed that while 57% of the global population aged 15 years or older had abstained from drinking alcohol in the previous year, more than half of the population in the Americas, Europe, and Western Pacific consumed alcohol. The spectrum of alcohol use behavior is broad: low-risk use (sensible and in moderation), at-risk use (e.g., binge drinking), harmful use (misuse) and dependence (alcoholism; addiction; alcohol use disorder). The at-risk use and misuse of alcohol is associated with the transition to dependence, as well as many damaging health outcomes and preventable causes of premature death. Recent conceptualizations of alcohol dependence posit that the subjective experience of pain may be a significant contributing factor in the transition across the spectrum of alcohol use behavior. This narrative review summarizes the effects of alcohol at all levels of the pain system. The pain system includes nociceptors as sensory indicators of potentially dangerous stimuli and tissue damage (nociception), spinal circuits mediating defensive reflexes, and most importantly, the supraspinal circuits mediating nocifensive behaviors and the perception of pain. Although the functional importance of pain is to protect from injury and further or future damage, chronic pain may emerge despite the recovery from, and absence of, biological damage (i.e., in the absence of nociception). Like other biological perceptual systems, pain is a construction contingent on sensory information and a history of individual experiences (i.e., learning and memory). Neuroadaptations and brain plasticity underlying learning and memory and other basic physiological functions can also result in pathological conditions such as chronic pain and addiction. Moreover, the negative affective/emotional aspect of pain perception provides embodied and motivational components that may play a substantial role in the transition from alcohol use to dependence.

KEYWORDS

alcohol misuse, nociception, pain-associated alcohol dependence, neuroimmune interaction, pain pathways, c-FOS, hyperkatifeia

Introduction

In recognition of the diversity and complexity of pain revealed by recent clinical and basic science, the *International Association for the Study of Pain* (IASP) re-evaluated their widely adopted definition of pain and revised it to "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage." The difficulty of encompassing all aspects of pain in a single definition necessitated the inclusion of 6 bulleted notes for further consideration [1]. In this

narrative review of the pain system and alcohol use we incorporate the IASP definition and notes and make the following basic distinctions. The sensation of pain is the subjective (conscious) experience of pain in response to the biological detection of dangerous or potentially dangerous stimuli. The sensation of pain is referred to as *nociceptive-pain* rather than nociception since nociception and pain are related but not identical constructs. In contrast, the perception of pain is a subjective experience that is primarily a psychological process involving the brain's systematic analysis and interpretation of physical information concerning potentially dangerous stimuli (including nociceptive-pain) and tissue damage. The perception of pain, which from here on will be referred to simply as pain, is challenging to study because it involves biological, psychological, and social factors and is learned through life experiences.

Pain can function adaptively in the short term (acute pain) and long term (e.g., by inducing learned behavioral change) or maladaptively in a chronic manner. Chronic pain is not a single maladaptive entity but reflects a progression from different pathologies. N*europathic pain*, for example, which requires an injury diagnosis such as nerve trauma or stroke, emerges from adaptive changes that lead to a chronic painful syndrome. *Inflammatory pain* is an adaptive response that sensitizes a nociceptive neural circuit to increase nociceptive-pain, but dysfunction in this adaptive response is a likely contributor to the transition from acute to chronic pain conditions [2]. The upregulation of nociceptor ion channels induces spontaneous activity causing a persistent nociceptive-pain experience that motivates recuperative behavior. This *sensitization* of nociceptors results in increased sensitivity at the site of exposure to the noxious stimulus (*primary hyperalgesia*) and to the surrounding area (*secondary hyperalgesia*) and can also induce the sensation of pain from thermal or mechanical stimuli that are normally innocuous (i.e., *allodynia*) [3, 4]. Many other inflammatory signals also impact on nociceptors as downstream targets by inducing upregulation of ion channels including histamine, bradykinins, prostaglandin E2, nerve growth factor (NGF), and protons H+. Hypersensitivity of neural circuitry also occurs in the spinal and supraspinal circuits of the central nervous system (CNS) and by consensus is conceptualized as *central sensitization* [2, 5].

Pain is considered chronic when it persists or recurs beyond a usual recovery period of about 3–6 months or when associated with a chronic health condition (e.g., cancer) [6]. Because pain is a subjective and emotional response to a personal experience, reliable self-report measures are the best indicators of a person's pain experience. But as noted by the IASP council, the inability to communicate an expression of pain does not indicate the absence of pain in human or non-human animals. Several strategies are used to assess patients who are unable to self-report [7]. Measures of pain-like behaviors have been developed in preclinical animal models of nociceptive-pain and chronic pain [8].

Nociception and nociceptive-pain

Traditionally, nociception refers to the sensing of noxious (intense) stimuli impinging on the body from the external (e.g., skin) or internal (e.g., muscles, viscera) environment by the class of sensory neurons named "noci-ceptors" by Sir Charles Sherrington [9]. Nociceptors expressed by first-order sensory neurons of the spinal cord (dorsal root ganglion, DRG), for example, transduce signals—mechanical, thermal, or chemical—from the environment into neural information that is conducted to second-order neurons within the dorsal horn of the spinal cord for nociceptive processing. The relay of nociceptive information to the brain is necessary for the subjective (conscious) sensation of pain (i.e., nociceptive-pain), however nociception itself (i.e., nociceptor activity) is not sufficient for the sensation of pain nor necessary for the perception of pain. Indeed, Sherrington introduced the concept of nociception to account for the skin's "special sense of its own injury" and the discovery, in an experimental spinal dog preparation, that a reflexive defensive withdrawal response continues to be elicited despite the separation of the spinal cord from the brain [10]. The dissociation of nociception from the sensation of pain is also evident in non-experimental contexts. For example, cough is a nociceptor-driven response that is not typically accompanied with nociceptive-pain [11, 12]. Nociceptor activation also plays a role in the protection against muscle injury under normal behavior repertoires by triggering innate motor patterns through spinopallidal circuits independent of the neural circuitry necessary for the cognitive or affective components of pain [13].

In animals, nociception and nociceptive-pain are assessed and inferred, respectively, using several accepted stimulus-dependent tests (see [8]). For humans, the *nociceptive flexion reflex* (NFR) is a popular objective neurophysiological tool for the assessment of nociception and nociceptive-pain. This polysynaptic reflex is activated involuntarily by noxious stimuli applied to a limb causing a protective withdrawal response. Because the NFR is moderately positively correlated with verbal reports of pain this measure is also used as an indicator of nociceptive-pain [14]. However, there are reports of the dissociation between the NFR and nociceptive-pain under clinically relevant (e.g., chronic pain syndromes) and normal situations [15–17]. It has also been shown under experimental contexts that stimulus-dependent withdrawal reflexes are influenced by cognitive and emotional factors modulating descending control of spinal circuits [18].

Our understanding of nociception as a defensive bodily response that is separate, although often concurrent with, nociceptive-pain has expanded remarkably by findings that nociceptors engage the immune system directly in defensive barrier functions and disturbances in homeostasis [19, 20]. A recent study utilizing newly developed optoelectronic technology confirms that nociceptor activation is sufficient to directly induce activation of innate and acquired immune cells [21]. The role of direct neural activation of immune function in response to physical insult, known as *neurogenic inflammation*, has long been recognized [22–24]. A well-established mediator of neurogenic inflammation are nociceptors that release calcitonin gene-related peptide (CGRP) and substance P antidromically to induce endothelial and smooth muscle cells to produce vasodilation, increased vascular permeability, and edema, resulting in the experience of redness, heat, and swelling at the site of injury. As first noted at the start of the first millennium A.D. by the Roman encyclopedist Aulus Cornelius Celsus, nociceptive-pain (dolor) accompanies the other three symptoms of inflammation -rubor, calor and tumis, respectively [25]. However, even when nociception is experienced as a sensation (i.e., nociceptive-pain), it is not just a symptom of bodily harm. Nociceptors are actively engaged in the regulation of inflammation by sensing pathogens and contributing to inflammation and the subsequent recovery of homeostasis.

Neuro-immune interaction in defensive action, homeostatic recovery, and maintenance is incompletely understood. For example, a recent study calls attention to our gaps in understanding of neuroimmune processes in the treatment of acute pain and the transition of acute pain to chronic pain. Treatment with steroidal and non-steroidal anti-inflammatory drugs for early musculoskeletal pain conditions have hypoalgesic efficacy, however early anti-inflammatory treatment interfered with a protective effect of acute inflammatory responses against the development of chronic pain in the long-term [26]. As is discussed below, a similar paradoxical effect is seen with alcohol. In animals and humans acute alcohol consumption has hypoalgesic properties,[1] but when alcohol consumption transitions to chronic consumption it hastens the progression to chronic pain a condition that is highly comorbid with alcohol misuse and Alcohol Use Disorder (AUD) [27]. A spotlight on the impact of different degrees of alcohol consumption on nociception, nociceptive-pain, and chronic pain may yield insight into neuroinflammatory processes and chronic pain and their role in the development and maintenance of alcohol misuse and AUD [28].

1 The terms analgesia and antinociception are often used synonymously with hypoalgesia, however the former is more appropriately defined as the absence of pain in response to a stimulus that would otherwise be subjectively experienced as painful whereas hypoalgesia and antinociception refer to diminished pain.

Molecular aspects of nociception

Nociceptors that result in intense short-term nociceptive-pain (sometimes referred to as "primary pain") are fast-acting myelinated (Aδ) neurons. Slow unmyelinated neurons (C) transmitting diffuse signals are experienced as dull, prolonged nociceptive-pain. The primary neurotransmitter is the excitatory neurotransmitter glutamate, but nociceptors are also modulated by several endogenous peptides at their peripheral and central terminals [29]. The molecular mechanisms of nociceptors are highly heterogenous. Nociceptors express many ion channels including specialized voltage-gated sodium channels ($Na_v1.7$, $Na_v1.8$, and $Na_v1.9$), mechanosensitive Piezo ion channels (Piezo1, Piezo2) and the transient receptor potential (TRP) channels. Among the latter are the TRP vallinoid 1 (TRPV1) and TRP ankyrind 1 (TRPA1) ligand-gated channels, also known as the capsacin receptor and the wasabi receptor, respectively. In addition to the sensing of mechanical (Peizo), temperature (TRPV1), and chemical (TRPA1) *danger* signals, nociceptors also detect *damage*-associated molecular patterns (DAMP) released from damaged tissue. DAMPS bind to pattern-recognition receptors such as Toll-like receptors (TLRs 3, 4, 7 and 9), signaling the innate immune system to promote a non-infectious inflammatory response [19]. For example, the chromatin-associated protein HMGB1 (high mobility group box 1) when secreted into the extracellular environment functions as an inflammatory cytokine. The binding of HMGB1 to TLR4 generates reactive oxygen species (ROS) and the downstream activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to induce proinflammatory gene activation [30, 31]. HMGB1 activation of NF-κB and ROS generation is also mediated through the stimulation of the receptor for advanced glycation end products (RAGE). Another major DAMP is ATP (adenosine triphosphate) detected by immune cells and nociceptors because of the expression of purinergic receptors on both cell types. Alcohol contributes to peripheral and central pain processing by directly inducing the release of DAMPS as a result of the toxic effects of the alcohol degradation product acetaldehyde and its byproducts or by impacting on DAMP mediated inflammatory reactions induced by other physical damages [32].

In addition to DAMPS neuro-immune interaction may be disrupted by gut-derived pathogens [22, 33]. Nociceptors detect microbial pathogens through pathogen-associated molecular patterns (PAMP) (e.g., LPS, flagellin, peptidoglycans) and bacterial products (e.g., N-Formyl peptides). Although gut microbiota is well established as a modulator of visceral pain, substantial evidence is accumulating that gut microbiota also play a role in many types of chronic pain, including inflammatory and neuropathic pain, by impacting on the peripheral and central nervous system [34].

The ability of nociceptors to detect pathogens and modulate the experience of pain through bidirectional neuroimmune integration reflects the broader ability of sensory neurons to interact with the microbiome, including symbiotic

(or commensal) microbiota to form a microbiota-gut-brain axis (for review see [35]). A role of symbiotic microbes in the causal mediation of nociceptive-pain has been confirmed by the experimental construction of axenic or "germ-free" mice made free from all microorganisms by preventing natural colonization by microorganisms. Behavioral measures of nociception in germ-free mice indicated reduced nociceptor sensitization to experimentally induced inflammatory signals which was reversed with restoration of microbiota using fecal transplants from conventional mice. Additionally, the commensal microbiota may have restored nociceptor sensitization by stimulating toll-like receptors [36].

Nociceptors are also modulated by the immune cells of the innate and acquired immune systems under pathological conditions resulting from tissue injury and infection by responding to molecular modulators including cytokines (tumor necrosis factors [TNF], interleukins (IL), interferons [IFN], chemokines [e.g., CCL1, CCL2], transforming growth factor [TGF], and prostaglandins (e.g., PGE2) [37]. These molecular modulators of nociceptive processing occur at all levels of the pain system including the peripheral nervous system (peripheral nociceptor terminals, dorsal root ganglion) and central nervous system (spinal cord, supraspinal brain circuits) [38]. Alcohol can alter these processes by producing dysbiosis of the gut microbiome which then impacts on peripheral nociceptors and the gut-brain communication through several pathways including through the vagus nerve [39, 40].

It is important to note that most of these studies, as with studies on pain and alcohol use and dependence in general, have been conducted with male subjects. More recent evidence, although limited, provide compelling evidence that there are sex differences in neuroimmune signaling and synaptic function as well as the disruptions that occur following chronic alcohol consumption. Sex differences can be seen in studies on transcriptomic analyses, cytokine regulation of the innate and acquired immune system, and regulation of alcohol intake by astrocytes and microglia (for a detailed review see [41]). Research on biological sex-dependent neuroimmune mechanisms is likely to provide insight into the relationship between gender and pain such as why woman have more experiences with perceived acute pain and show greater prevalence of some forms of chronic pain (e.g., fibromyalgia) [42]. Moreover, changes during aging in pain sensitivity, chronic pain, and the role of molecular mechanisms including via neuroinflammation is still not well characterized [43].

Alcohol, nociception, and nociceptive-pain

Numerous experiments in animals convincingly demonstrate that forced administration or voluntary consumption of alcohol has short-term hypoalgesic properties as indicated by raised nociceptive thresholds in response to thermal stimuli (tail flick and hot-plate tests) and other measures of nociceptive-pain and allodynia (e.g., Von Frey mechanical sensitivity) (for review of different methods to measure nociceptive-pain in rodents see [44] and for the effects of alcohol on these measures see [45]). For example, Gatch and Lal [46] showed that alcohol administered to rats acutely (i.p.) induces hypoalgesia (dose-dependently) and when given chronically in a liquid diet. Although the hypoalgesic effect of chronic alcohol shows tolerance, withdrawal of alcohol induces hyperalgesia that is reversed by re-administration of alcohol. Withdrawal-induced hyperalgesia and mechanical allodynia is also seen when alcohol is given as a chronic intermittent ethanol vapor although the effects are moderated by several factors including amount of alcohol exposure and sex [47–49]. Protocols using intermittent chronic alcohol exposure in rodents have been used successfully as reliable and valid animal models of drug and alcohol dependence. Preclinical studies on chronic pain and AUD provide new insight into the reciprocal influences between the common morbidity of pain and alcohol dependence and potential treatment strategies [45].

Alcohol can also have robust dose-dependent analgesic properties in healthy human volunteers experiencing experimentally induced nociceptive-pain [50, 51]. Although experimental nociceptive-pain differs in many ways with clinical pain, there is evidence that the analgesic properties of alcohol may support self-medication behaviors of pain sufferers. Experimental induction of a moderate but clinically significant acute pain (capsaicin plus heat) increased the urge and intention to drink alcohol in healthy undergraduate students reporting frequent drinking experiences [52]. Several studies have reported an association between moderate alcohol use and reduced pain especially in men [51, 53, 54]. A recent ecologically relevant experimental study investigating behavioral economic measures of the self-medicating use of alcohol following induced delayed musculoskeletal pain (i.e., a common experience of delayed onset muscle soreness that occurs after exertion) revealed an increased demand for alcohol in males, although a decreased demand in women [55]. The hypoalgesic effects of alcohol consumption can also be observed despite the presence of chronic pain [56]. Paradoxically, as discussed further below, alcohol may be an effective hypoalgesic for the short-term relief of pain but long-term consumption of alcohol results in exacerbated pain, increasing an individual's risk towards alcohol misuse and the development of AUD [51]. The sex specific effects in these studies support existing research highlighting sex (biological) and gender (psychosocial) differences in pain perception and tolerance [41, 57] and suggest that men are at increased risk of developing AUD when self-medicating for nociceptive-pain, despite many studies indicating that females are disproportionally affected by chronic pain [58].

Neuroimmune interactions and alcohol

The regulation of the immune system is intricate and made even more complex by its bidirectional communication with the nervous system. The complexity of alcohol's modulation of these functions reveals itself in paradoxical ways. It is clear that alcohol modulates innate immunity to microbial products in a dose- and time-dependent manner, although the relationship among these variables is inconsistent in the literature most likely due to differences in methodology and parameters. While most studies are based on *in vitro* experiments, *in vivo* studies confirm opposing effects of alcohol exposure on the inflammatory response of innate immune cells. For example, while short-term exposure (hours) reduces levels of systemic inflammation, long–term exposure (days) stimulates proinflammatory cytokines and decreases anti-inflammatory cytokines [59]. Similar temporal differences of drinking on immune function may explain observations that light to moderate drinking improves responses to vaccines, but heavy chronic drinking is associated with immune dysfunction [60]. It is unclear if alcohol's hypoalgesic effects in short-term drinking and hyperalgesia in chronic drinkers reflect this paradoxical effect of alcohol on immune function.

The initial impact of alcohol following its consumption is of course on the gastrointestinal system, being absorbed mainly in the upper intestines and entering the blood circulation and the portal circulation to the liver. The presence of ethanol in the blood also serves to maintain persistent levels of alcohol throughout the gastrointestinal tract until alcohol is eliminated through several metabolic pathways. The most relevant pathway in light to moderate drinkers is the metabolism by alcohol dehydrogenase (ADH) in the liver into the toxic compound acetaldehyde potentially causing hepatocyte injury and the release of DAMPS. Aldehyde dehydrogenase (ALDH) then metabolizes acetate into a less toxic compound, which is then metabolized to Acetyl CoA, a product that is also a key metabolite of the major nutrients—carbohydrates, fat, and protein. Another pathway, especially in heavy drinkers, is Cytochrome P450 2E1 (CYP2E1) which results in ROS contributing to oxidative stress [32, 61]. Studies in humans and animals demonstrate that in the presence of chronic alcohol exposure there are increases in bacterial loads and in the permeability of the gastrointestinal barrier allowing bacteria of the microbiome and their endotoxins (i.e., lipopolysaccharides, LPS) to enter the bloodstream [62]. Preclinical studies with rodents show that a "leaky gut" due to repeated cycles of alcohol exposure increases the release of LPS which affects peripheral and brain immune (i.e., microglia) signaling that may also lead to the progression and persistence of problematic alcohol use behavior [30, 39]. As discussed later, the role of pain in alcohol misuse and AUD has become an important area of interest [63]. Because LPS also acts directly on TRPA1 channel of nociceptors to induce a rapid modulation of nociception and nociceptive-pain, a "leaky gut" may contribute to the progression and maintenance of maladaptive alcohol use by modulating alcohol-associated nociception, nociceptive-pain, and chronic pain [64]. This possibility needs to be further investigated.

Thus, nociceptor component of the pain system and the immune system share the role of detecting acute perturbations in homeostasis due to noxious stimuli and potentially pathogenic microbes and engage in integrated protective countermeasures—from adjustments in behavior (to minimize tissue injury and to escape and avoid dangerous stimuli) to the neutralization of pathogens, resolution of inflammation, and the restoration of tissue homeostasis. Nevertheless, given that the neuron-immune integration to dangerous and damaging stimuli is varied and extremely complex, it is not surprising that these processes can become dysfunctional leading to failed or maladaptive homeostasis resulting in disease processes such as chronic pain. The role of alcohol in these neuro-immune processes as it relates to pain is understudied. Furthermore, nociception needs to be viewed more broadly, not simply as the direct initiator of nociceptive-pain and the perception of pain but in a broader context of neuro-immune regulation and possible alcohol-induced dysfunction of homeostasis and allostasis.

Spinal and supraspinal circuit structures

The seminal gate control theory of pain shifted pain research from the Cartesian view of the brain as a passive receiver of pain signals presumed to be generated in damaged tissue to the current understanding of the central nervous system as the dynamic source of pain [65]. Melzack [66] further developed the idea of the centrality of pain by theorizing the "neuromatrix" as a neural network integrating sensory-discriminative (e.g., nociceptive-pain), affective-motivational, and evaluative-cognitive dimensions in the construction and embodiment of pain experience. Several decades of empirical research continues to strongly support this explanatory model and paradigm shift in pain research. Figure 1 shows several of the components of the pain system identified in this review as it relates to Melzack's conceptualization of the "neuromatrix".

Nociceptors are dorsal root ganglion neurons in the peripheral nervous system that project to the dorsal horn of the spinal cord with myelinated (A-Delta fibers) or unmyelinated (C-fibers) axons, synapsing with secondary neurons in Rexed laminae I and II of the gray matter (also known as the marginal zone and substantia gelatinosa, respectively) and connecting with interneurons, descending modulating neurons, and afferent neurons in other laminae. The neurotransmitters involved in excitatory interactions include glutamate and substance P, while inhibitory neurotransmitters include GABA. The secondary neurons cross the midline and project to supraspinal structures via two primary paths through the thalamus as part of the anterolateral system (except for nociceptors of the face which follows a separate route to the thalamus via the trigeminal nerve). The sensory-discriminative

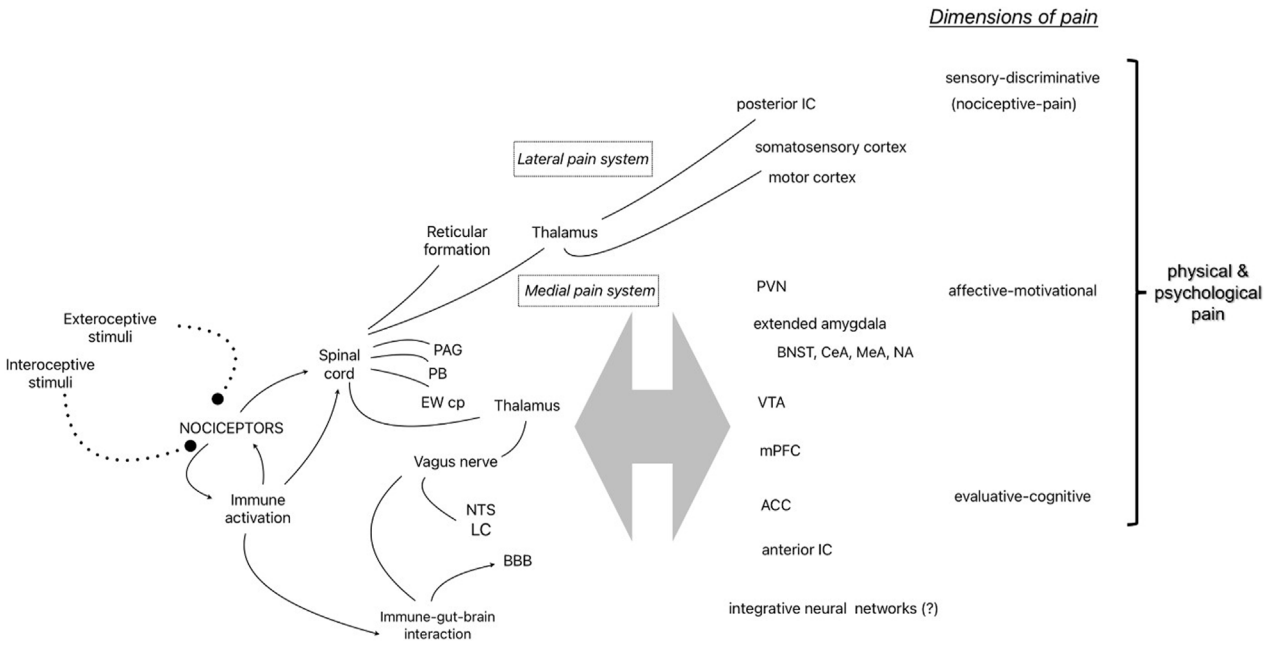

FIGURE 1
Representative components of the pain system identified in the narrative review and the 3 dimensions of physical and psychological pain [55]. Abbreviations: PAG, periaqueductal Gray; PB, parabrachial nucleus; EWcp, centrally-projecting Edinger-Westphal nucleus; NTS, nucleus of the solitary tract; LC, locus coeruleus; BBB, blood brain barrier; IC, insular cortex; PVN, hypothalamic paraventricular nucleus; BNST, bed nucleus of the stria terminalis; central (CeA) and medial (MeA) nucleus of the amygdala; NA, nucleus accumbens; VTA, ventral tegmental area; mPFC, medial prefrontal cortex; ACC, anterior cingulate cortex.

dimension (e.g., nociceptive-pain) is attributed to the lateral pain system which includes the spinothalamic tract carrying information to tertiary neurons in the lateral thalamic nuclei that project to the posterior insular cortex and primary somatosensory cortices to provide information about location and intensity of nociceptive stimulation, while some ascending fibers make direct connections with the reticular formation in the brainstem (spinoreticulothalamic tract), possibly to direct attention to nociceptive stimuli. A separate medial route carries information to the periaqueductal gray (PAG) and the parabrachial nucleus (PB) at the junction of the pons and midbrain in route to the amygdala and other forebrain structures attributed to the affective-motivational and evaluative-cognitive dimensions of the neuromatrix [67, 68]. Interestingly, experiments with decerebrate animals which remove the integration of forebrain structures with the hindbrain by surgical separation of the connection with the brainstem and spinal cord have demonstrated intact escape-like behaviors (i.e., nocifensive behaviors) to specific noxious stimuli [69]. The PB is one critical structure receiving nociceptive input that appears to diverge into at least two distinct pathways. One neural pathway for the direct activation of nocifensive behavior (via the ventromedial hypothalamus or lateral PAG) and another pathway for the experience of pain and learning involving the forebrain structures, bed nucleus of the stria terminalis (BNST) or central nucleus of the amygdala (CeA) [70]. Thus, ascending nociceptive information (along with descending modulating influences) is integrated at many levels of the neuroaxis resulting in neural pathways that mediate many nociception-related functions - from the activation of nocifensive behaviors to the integration of nociceptive information with affect, emotion, cognition and learning [71]. It is the latter integrative function that transforms nociceptive information from a basic sensory experience (Melzack's sensory-discrimination dimension) to a constructed perception that is experienced as pain [66, 72].

Supraspinal structures involved in affective-motivational aspects of pain

Supraspinal pain pathways include complex circuitry classically associated with affect/emotion[2] and reward which are also critical contributors to drug and alcohol misuse and dependence according to some extant theoretical models of

2 Affect, mood, and emotion are subjective terms that are not consistently differentiated from one another. Affect is more typically defined as a broad range of subjective experiences that vary in terms of valence (positive to negative) and level of arousal. Emotion and mood are considered distinct phenomena, with the former typically short in duration and directed at a stimulus source.

addiction (see [73]). These shared circuits may also explain why world-wide across many cultures the same words (e.g., "pain" and "hurt") are often used to describe seemingly different experiences such as actual physical injury (i.e., nociceptive-pain) and rejection from a social partner (social or psychological pain) [74]. In one functional MRI study the experimental induction of nociceptive-pain and social pain in the same participants activated the same cortical structures indicating that the two negative emotions shared similar somatosensory representations [75]. Other functional imaging studies provide support for the similarity and shared forebrain structures between nociceptive-pain and social pain [76]. Among the forebrain structures shared by these is most notably the amygdala and its various inputs and outputs.

The amygdala—consisting of 3 nuclear groups, the basolateral amygdaloid complex (BLA), central nucleus (CeA), and cortical-like group (Co)—is well suited for the integration of sensory/perceptual and affective/emotional information. The main subregion receiving extrinsic (sensory) information, including nociceptive information, is the BLA group which consists of the lateral nucleus (LA), basolateral nucleus (BL), and basomedial nucleus (BM). The LA is the primary input region receiving projections from higher order sensory association areas of the cortex and connecting reciprocally with the other BLA nuclei and other amygdala groups [77, 78]. The BLA also receives projections from the medial prefrontal cortex (mPFC) which provides affective (or valence) information and emotion-based information modulated by executive functions (e.g., decision making) to guide behavior and action [79]. The primary neurotransmitters in the amygdala circuitry is glutamate involving excitatory transmission and GABA involving tonic and phasic inhibitory transmission [80]. The major output of the amygdala complex is the CeA which has extensive projections to the lateral hypothalamus, basal forebrain regions, and brainstem. The CeA also forms a circuit referred to as the *extended amygdala* hypothesized to be involved in evaluating the affective value of sensory stimuli.

The concept of the *extended amygdala* was first introduced a century ago when comparative developmental neuroanatomy studies determined that the CeA forms a continuous pathway with the BNST in vertebrates [81]. Extensive experimental studies demonstrating the key role of the extended amygdala in the acquisition and expression of fear memories has established these structures as key components of an emotion brain circuit [82]. In adult mammals the extended amygdala consists of the BNST, central and medial amygdalae (CeA, MeA), and a transition zone in the shell (medial portion) of the nucleus accumbens (NA). These structures are not only important in emotion, but are also involved in learning, memory and reward processes that allow emotion to be integrated with perception, learning, memory, and behavioral action. The recruitment of the extended amygdala is hypothesized to play an important role in the multistage model of alcohol dependence [73]. With respect to pain, structures such as the CeA serve to integrate nociceptive information and modulate the perception of pain through its outputs to the forebrain, brainstem and spinal cord. Most of the brain pathways associated with pain have been elucidated using rodents primarily, but with support from human neuroimaging studies. Correlations between amygdala activity and pain-like responses in rodents, and pain verbal reports in people, have been widely reported [50, 79, 83, 84]. The involvement of the CeA neurons in nociceptive and pain-related processing possibly via input from the paraventricular nucleus of the thalamus, has been described as the "nociceptive amygdala" [85]. The CeA has also been described as an "integrative hub" for negative affect (e.g., anxiety) and alcohol use disorders [80]. Nociceptors project axons to the CeA through the parabrachial (PB) nucleus providing information about a range of homeostatic functions including information about noxious stimulation [86]. The PB also receives "top-down" descending pain modulatory signals [87]. Dysregulation of excitatory and inhibitory neural activity may result in neuropathic pain as suggested by optogenetic studies with mice experiencing experimentally-induced hypersensitivity to aversive stimuli [88]. For example, hyperalgesia induced by alcohol withdrawal in alcohol-dependent rats is mediated by CeA projections to the ventrolateral PAG neurons containing μ-opioid receptors. The CeA distributes GABAergic neurons to these PAG neurons to inhibit the perception of pain, but in rats experiencing withdrawal in an alcohol vapor model of alcohol dependence the inhibitory CeA signals were weakened thereby facilitating nociception signals and likely leading to increased nociceptive-pain (i.e., hyperalgesia) [89]. Recent work also implicates changes in dopamine-, melanocortin- and corticotropin-releasing factor signaling in the reciprocal relationship between the midbrain and CeA that may be moderated by sex and age [90].

Affective-emotional brain structures and alcohol

A role for several of these same forebrain structures in alcohol consumption was first implicated by Chang et al [91] when rats treated with intraperitoneal injections of alcohol showed dose-dependent increases in the immediate early gene, c-FOS, activation (a marker of neuronal activity) in the PB, BNST, and CeA as well as the Edinger-Westphal nucleus (EW), paraventricular hypothalamic nucleus (PVN), and locus coeruleus nucleus (LC).

The Chang et al [91] study was complemented by the finding that alcohol is as effective as LiCl to induce conditioned taste aversion and an associated increase of FOS expression in the PB [92].[3] The PB is now well known to be a crucial structure for

3 Lithium chloride (LiCl) is a compound commonly used to establish conditioned taste aversions in preclinical studies using animals.

conditioned taste aversion - an important learned behavioral strategy to defend homeostasis by avoiding subsequent exposure to previously consumed life-threatening substances [93, 94]. Similar alcohol-induced FOS expression have been found in different mouse strains genetically selected to engage in high levels of alcohol self-administered while engaged in different patterns of intake, although strain differences in c-FOS activation were observed in other brain regions associated with ethanol drinking [95]. While these FOS immununoreactivity studies confirmed that the PB at the very least receives information concerning the presence of aversive systemic alcohol, subsequent studies demonstrated a role of the PB in modulating alcohol consumption. For example, optogenetic stimulation of neurotensin neurons projecting from the amygdala to the PB increases the intake and rewarding value of alcohol and other palatable solutions [96].

These findings indicate that the PB is involved in the aversive and rewarding properties of alcohol. Although alcohol exposure (by experimental treatment or self-administration procedures) is initially aversive, the aversive properties decline with repeated exposure to ethanol and the rewarding properties increase. Indeed, c-FOS activation following acute ethanol administration causes c-FOS activation to decline (desensitize) in the PB and other alcohol-sensitive brain structures at different rates with the EW showing more sustained sensitivity than the other nuclei [91].

The earliest studies demonstrating sensitivity to alcohol in the Edinger-Westphal nucleus in the brain stem was surprising because this structure was known to be a part of the oculomotor nuclear complex sending parasympathetic nerve fibers to the eye. However, the structure is now recognized to consist of distinct brain regions, a preganglionic EW nucleus projecting to the ciliary ganglion to regulate oculomotor function and a centrally-projecting nucleus (EWcp) that is highly sensitive to alcohol administration and projects to several brain regions including the BNST, CeA, dorsal raphe nucleus (DRN), anterior cingulate cortex (ACC), preoptic area (POA), medial prefrontal cortex (mPFC), lateral hypothalamus (LH), and ventral tegmental area (VTA). The EWcp neurons express several neuropeptides known to be associated with stress, reward, and administration of drugs of misuse, including urocortin 1 (Un1), cocaine and amphetamine-regulated transcript (CART) and substance P [97, 98]. There is also evidence from animal studies that the EWcp is activated in response to nociceptive stimuli and is possibly involved in chronic pain. A cluster of EW neurons with colocalized cholecystokinin (CCK) and substance-p in rats increases its firing rate in response to nociceptive simulation (toe-pinch). This neuronal activation is suppressed by systemically administered morphine—an effect reversed by naloxone [99]. Noxious visceral stimulation of the EW in rats increased expression of immediate early genes including c-FOS [100].

Interest in the role of EW in stress, pain, and alcohol consumption increased with the discovery of Un1 neurons [97]. Un1 belongs to the CRF neuropeptide superfamily, the principal hypothalamic stress-related neuropeptide, and binds with CRF-1 and CRF-2 receptors to induce G-protein-coupled signaling. The EWcp has the largest population of Un1 neurons often colocalized with CART. The EWcp projects to many sympathetic-innervated targets in the brainstem and spinal cord and has been proposed to function as a central orchestrator of the sympathetic nervous system's response to stress [97].

Interestingly, pain, stress, and alcohol induce a delayed and more sustained neural activation in the EW compared to other brain nuclei. As a comparison, for example, corticotropin releasing factor (CRF) releasing neurons in the PVN show more immediate and transient activation following an acute stressor stimuli [101]. Sustained activation of Un1 neurons also occurs following acute formalin-induced nociceptive-pain and chronic ether stress [97, 102]. These and other findings have led to the hypothesis the EWcp plays a critical role in adapting to bodily perturbations caused by acute stressful events, physical injury (nociceptive-pain), and ingestion (including potentially dangerous compounds such as alcohol) [97, 101, 103]. Thus, the EWcp is a likely key player in energy metabolism and the defense of homeostasis (for review see [101]). However, chronic stress, repeated pain experiences, and any associated alcohol and drug use may disrupt the return to homeostasis causing an allostatic shift (i.e., the establishment of a new homeostatic state) and the emergence of enduring, relapsing conditions such as chronic pain or the behavioral changes seen in the addiction phenotype [101, 104].

Homeostasis and allostasis

Occasional acute physical disturbances or infrequent experiences that may be a potential threat (stressor) result in an adaptive protective response followed by the return to a static but "normal" homeostatic function. Homeostasis makes sense within a physical system that maintains stable features to match an environment that is unchanging notwithstanding irregular and temporary perturbations. However, with the emergence of a chronic environmental stressor or persistent repeated exposures to physical insults the maintenance of a "normal" homeostatic baseline no longer makes sense. To adapt to these new persistent environmental demands allostatic processes are engaged that predict the optimal physiological parameters needed to achieve stability [105]. Thus, unlike homeostasis which maintains optimal parameters within steady state "normal" levels, allostasis is a dynamic whole-body process involving the prediction of optimal levels of functioning based on anticipated demand from changing environmental variables. In essence, the body is learning to adapt to changing environmental demands. Although allostasis reflects efficient physiological regulation, current allostatic models of disease conceptualize the gradual life-time buildup of "wear and tear"

of the body (or allostatic load) as causing the overactivation or dysregulation of allostatic systems that mediate the effects of chronic stress on disease and mental health [105, 106]. This concept of maladaptive allostasis in brain stress systems have also been advocated to explain addiction and possibly chronic pain.

According to the multistage model of the development and maintenance of alcohol addiction proposed by Koob et al., stress and reward systems undergo changes to maintain hedonic stability in an allostatic state [73, 104, 107]. However, the buildup of allostatic load may progress an individual towards alcohol misuse and addiction in 3 stages of motivated behavioral change: 1) binge and intoxication driven by positive reinforcement, 2) withdrawal and negative affect relieved through negative reinforcement, and 3) preoccupation and anticipation with the drug of choice that is mediated by associative learning (i.e., Pavlovian conditioning).

Maladaptive allostasis in addiction emphasizes the role of emotional states in guiding motivated behavior. Initially alcohol may provide a pleasant affective/emotional experience. It may be positive reinforcement (or reward) due to the pleasant experience of the alcohol consumption ("a buzz") or the social approval of drinking in the presence of others. Or it may be negative reinforcement as a result of the temporary reduction of an unpleasant experience such as transient relief of physical or psychological pain. In either case the exogenously administered alcohol induces a departure from homeostasis and thus the body will address the temporary alcohol-induced perturbations (no matter if the effects are willfully wanted or unwanted by the individual) by activating opponent-like processes that counteracts the drug as well as the concomitant affective/emotional change [104, 108]. According to an allostatic perspective repeated exposure to alcohol intake (interacting with genetic factors, unique life experiences and psychiatric co-morbidities) can result in maladaptive allostasis leading to pathological states such as alcohol dependence. Koob proposed the psychological construct of hyperkatifeia, an exaggerated negative emotional state (i.e., increased psychological pain and distress) that can occur during periods of alcohol withdrawal to maintain addictive behavior through craving and negative reinforcement [63]. This heightened emotional state has a parallel in the pain system in the form of the transition from alcohol-induced analgesia to alcohol-induced hyperalgesia and chronic pain [109].

Self-medication with alcohol

Before discussing self-medication with alcohol, it is worth nothing that acute and chronic consumption of alcohol has many potential injurious effects on the body. The largest area of investigation has been on the role of chronic alcohol misuse on the burden of preventable diseases of the liver, pancreas, and gastrointestinal tract [110]. Clinical studies and preclinical models indicate that females experience greater harms from alcohol despite drinking less than males, yet the gap in alcohol consumption between men and women is now narrowing (e.g., [111–114]). Some of the deleterious effects of chronic alcohol misuse and addiction are due to consequent nutritional deficiencies. Chronic alcohol consumption often leads to reduced intake of dietary thiamine (Vitamin B1) which is further exacerbated by alcohol-induced malabsorption of this essential vitamin. Thiamine deficiency interferes with several critical cellular functions resulting in toxic effects on several brain regions leading to disorders of the brain such as Wernicke-Korsakoff syndrome and to neuropathies of the peripheral nervous system [115]. More direct mechanisms of neuropathic pain caused by alcohol or its metabolites have been proposed and are active areas of investigation; for example, oxidative stress nerve damage due to overproduction of ROS, sustained activation of the hypothalamic-pituitary-adrenal (HPA) axis, overactivation of protein kinase C (PKC), and dysregulated neurocircuitry are just a few examples of possible mechanisms [116]. Excessive misuse of alcohol is also causally associated with neurodegenerative disorders (e.g., Huntington's disease, generalized dementia, multiple sclerosis) and some types of cancers (e.g., upper alimentary tract and liver) [117, 118]. Acute effects of alcohol can also induce different degrees of injurious outcomes. Research implicates neuroinflammation involving TLR4 and TRPV1 in the transient effects of alcohol-induced headaches experienced by some people when drinking fermented beverages [119]. Acute but excessive amounts of alcohol may also interfere with the innate immune system defense against bacterial infection by injuring hematopoietic tissue and impairing bone marrow production of granylocytes (including neutrophils, eosinophils, and basophils) increasing vulnerably to bacterial infection and sepsis [120]. And, of course, intoxicating levels of alcohol increases vulnerability to engage in risky behaviors that can result in highly injurious outcomes leading to long-term pain and disability as well as loss of life. In a recent meta-analysis, 27% of fatalities from non-traffic injuries were attributable to misuse of alcohol [121].

Notwithstanding our current knowledge of alcohol misuse as a leading risk factor for disease burden, since antiquity there has been an enduring belief in the medicinal power of alcohol. Evidence indicating a complex association between alcohol use and health includes several decades of evidence for the protective benefits of moderate alcohol use on cardiometabolic health, for example, [122, 123]. More recently, there has been an increase in caution expressed about the view that alcohol-in-moderation yields health benefits. Despite the promising results of many short-term randomized controlled studies, this concern over the presumed health benefits of alcohol is based on the lack of long-term randomized trials of moderate alcohol consumption compared with no (or very low) alcohol drinking [124]. As a result, interest in Mendelian randomization (MR) studies has

grown in popularity. MR is an epidemiological method that mimics a randomized long-term controlled setting to establish possible causal relationships in observational data [125]. In conventional observational studies the presence of a causal relation between alcohol consumption (a potential cause) and a protective health outcome is limited by the possible presence of confounding variables, reverse causation, and measurement error. In a Mendelian randomization design genetic variants (e.g., ALDH2 polymorphic gene) that are reliably associated with different levels of exposure to a potential causal factor (e.g., alcohol consumption) but uncorrelated with the outcome of interest (e.g., cardiovascular disease) is analyzed to estimate a true causal effect between the potential causal factor and the outcome (if any). However, MR studies that have investigated the effects of alcohol drinking on cardiovascular health have been inconsistent suggesting that further studies are needed for refinement of MR and integration with other research methods [125–129]. Nevertheless, there is substantial evidence in humans and rodents that acute consumption of alcohol can be motivated by the experience of efficacious self-medication. The self-medication hypothesis is a causal model that posits that individuals drink alcohol under aversive conditions as a way to cope with anxiety, depression, and pain (i.e., negative reinforcement).

Support for the self-medication model comes primarily from studies investigating self-medication as a contributor to abusive alcohol use comorbid with anxiety and depressive disorders [130–133]. That is, as drug or alcohol use becomes a more frequently relied upon as an efficacious coping strategy, the use can transition to problematic use and addiction. Laboratory experiments also demonstrate the effectiveness of alcohol consumption in reducing experimentally induced stress, although these effects may rely on the influence of prior experience and the type of stressor [134, 135]. Preclinical studies demonstrate that rodents will self-medicate with alcohol and some anxiolytics when experiencing aversive emotional states (psychological pain) induced by loss or reduction of expected reward. For example, rats show a greater consumption of alcohol over water immediately after an expected highly preferred reward is omitted or reduced to a less preferred value [136–138]. Interestingly, reward loss also induces a reduced sensitivity to nociceptive-pain (hypoalgesia) which appears to reflect activation of a compensatory opioid and cannabinoid system to modulate physical and psychological pain as a component of homeostatic and allostatic modifications [74]. It is clear that low or moderate amounts of consumed alcohol also exerts clinically relevant hypoalgesic effects in controlled experimental studies with people and animals [50, 55, 56, 139]. Similar effects of alcohol and endogenous opioids on nociceptive-pain suggest an intersection of neural circuits, more specifically the opioid-mediated regulation of GABA neurotransmission [109, 140]. The possible involvement of alcohol's effect on inflammation and inflammatory cytokines acting on μ-opioid receptor regulation also needs further investigation [141].

Hypoalgesia and hyperalgesia

Paradoxically, while acute alcohol drinking reduces sensitivity to pain repeated administration of alcohol, like opioids and other analgesic drugs, results in greater sensitivity to physical nociceptive-pain-inducing stimuli (hyperalgesia). Evidence of opioid-induced hyperalgesia after chronic exposure to opioids is well established in preclinical studies and is observed in clinical populations particularly individuals with opioid use disorder [124, 142]. Chronic alcohol consumption results in neural alterations that are also seen in chronic pain—a decrease in inhibitory GABA activity along with hyperglutamatergic activity [109, 143, 144].

The transition from efficacious reduction in psychological and physical pain during acute alcohol administration to the opponent-like process of hyperalgesia appears to be exacerbated with repeated experiences of withdrawal from alcohol. Chronic voluntary alcohol consumption induces hyperalgesia in rats, an effect that further increases during periods of alcohol withdrawal [145, 146]. A recent experimental study also demonstrated alcohol withdrawal-associated hyperalgesia in young adult binge drinkers with only 1–3 years of drinking history [147].

The effects of alcohol withdrawal in animal models are particularly interesting. Early models of AUD required the time-consuming procedures to induce pharmacologically-relevant levels of alcohol in rodents and primates such as sucrose-fading procedure and scheduled-induced polydipsia. For example, sucrose-fading procedure exposed rats to mixtures of ethanol and sucrose to drive high levels of consumption followed by the gradual reduction of sucrose to zero [148, 149]. Many current studies use intermittent access to unadulterated alcohol often in binge-like patterns to elevate consumption in rodents (relative to continuous access). One possible mechanism of escalated drinking in intermittent procedures appears to be repeated periods of acute withdrawal, which would be accompanied with withdrawal-induced hyperalgesia and other aversive experiences. Interestingly, alterations in glutamate neurotransmission are consistently associated with intermittent procedures compared to rodents continuously exposed to alcohol [150].

Top-down construction and modulation of pain perception

It is well established in the field of perception that the experience of perception in any modality is influenced by top-

down cognitive processes determined by context or expectations and beliefs based on prior experiences. This includes pain perception. As already discussed, it is inadequate to view pain as a direct readout of nociceptive input. Early theories explaining pain in terms of direct dedicated pathways for nociception began to be questioned by paradoxical observations such as the observation of less than severe pain or no pain in soldiers with extensive wounds [151]. The phenomena of phantom limb (persistent sensations in a missing or amputated limb) and placebo hypoalgesia (pain relief from the expectation of a beneficial or therapeutic outcome) inspired Melzack to include the evaluative-cognitive dimension in the neuromatrix theory of pain perception [66]. Brain structures implicated in the cognitive modulation of pain include the anterior insular cortex (IC) and anterior cingulate cortex (ACC), structures shared with circuitry implicated in emotion, reward, and drug and alcohol addiction [73, 152]. The PAG along the caudal rostral axis of the midbrain is the most well-characterized pathway involved in descending pain modulation through its connection with the dorsolateral PFC, rostral ACC, hypothalamus, and ventromedial medulla, and spinal cord [71, 153]. Experimental human studies on placebo hypoalgesia and expectation effects show that the descending modulation of pain pathways are mediated primarily through endogenous opioids and dopaminergic signaling mediating negatively reinforcing pain relief or expectations of pain persistence, for example [154–156].

Placebo reduction of nociceptive processing at the level of the spinal cord shows the role of cognition in modulating nociceptive-pain at the level of sensory-discrimination dimension [153]. However, cognitive factors likely play more than a modulatory role in pain perception. As seen in other sensory modalities, top-down processing is fundamental to the construction of percepts resulting in individual differences in perceptions of the external world as revealed by ambiguous stimuli. As an example, consider the image of "the dress" that took the internet by storm in 2015 generating substantial interest among the public and the vision science community. Some observers perceived an overexposed image of a dress as black and blue—the actual color of the dress—while to others the dress appeared gold and white. People debating the dress color were incredulous—how can others looking at the identical image see colors that were undoubtedly wrong as informed by their own eyes? One possible explanation came from an empirical study showing that the ambiguous nature of the image required spontaneous assumptions about the source of lighting in the image for disambiguation, assumptions that differed depending on an individuals' prior life experiences [157]. Similarly nociceptive-related signals, possibly emerging at multiple levels of the pain system, and homeostatic/allostatic feedback may sometimes be ambiguous to the pain system. The uncertainty of pain and other harmless bodily sensations (see [158]) may be disambiguated by the individual's prior life experiences, expectations, and beliefs resulting in divergent interpretations and idiosyncratic experiences of pain. Contemporary perception research provides guidance on how to approach verbal reports of acute or chronic pain in the absence of evidence of tissue damage. Rather than dismissing such reports as "all in their heads" they should be treated as no less real than any other percept. The extent to which alcohol use, misuse and addiction contributes to ambiguity and disambiguation in the pain system and integrative neural networks may be a fertile area for investigation.

Discussion

In this narrative review, we aimed to present an overview of the current understanding of the mechanisms of nociception, the sensation of nociceptive-pain, and pain perception to inform and guide research on the contribution of the pain system in alcohol use, misuse, and dependence. Conventional wisdom influenced by the centuries-old Cartesian model of pain views physical hurt as a nociceptive experience that is directly translated into the sensation and perception of pain. However, it has become clear that nociception and pain are closely related but distinct mechanisms of homeostasis in defense against injury and potential injury. It will be useful to investigate the impact of alcohol use and misuse on the role of nociception in the direct defense against noxious stimuli and pathogenic microbes through its action on the innate and acquired immune system and bidirectional neuroimmune communication. Such studies are likely to provide insight into how these alcohol effects influence the sensation of nociceptive-pain and possibly how alcohol-induced effects impact on bottom-up inputs for the constructive perception of pain. The influence of alcohol use on nociceptive processes and nociceptive-pain may provide a better understanding of the paradoxical effects of repeated alcohol use such as the transition from alcohol-induced hypoalgesia to alcohol-induced hyperalgesia. The excessive use of alcohol may contribute to additional changes in the pain system resulting in the development of chronic pain and maintenance of the abusive alcohol use behavior by negative reinforcement processes perhaps further mediated by maladaptive homeostasis and allostasis, contributing to progression and maintenance of addiction (i.e., alcohol use disorder).

Brain structures involved in neural pain circuits are shared with pathways mediating emotion and reward, as well as neural circuits that play a role in psychological disorders associated with stress, fear, anxiety, depression, and drug and alcohol misuse and dependence. The complex interrelationship between neuroimmune interactions and the neural circuits and networks involved in negative emotion, pain, and drug use disorders suggest that the activation of pain circuitry may play

a role in the development and maintenance of alcohol dependence.

The role of reinforcement processes and top-down cognitive processes in the construction and modulation of pain perception (as well as gender-specific differences) validates the importance of identifying and establishing psychological approaches to prevent the transition of acute pain towards chronic pain, alcohol misuse, and alcohol addiction.

Author contributions

MV conducted the bulk of the writing and the crafting of the figure. SLC provided initial manuscript workflow and conducted reading, and editing and finish writing. MV crafted final manuscript layout and conducted reading, editing and finish writing. SLC and MV conceived the study. SLC and MV contributed to and approved the content of the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Epigenetic regulation of microglia and neurons by proinflammatory signaling following adolescent intermittent ethanol (AIE) exposure and in human AUD

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Adolescent alcohol drinking is linked to high rates of adult alcohol problems and alcohol use disorder (AUD). The Neurobiology of Alcohol Drinking in Adulthood (NADIA) consortium adolescent intermittent ethanol (AIE) models adolescent binge drinking, followed by abstinent maturation to adulthood to determine the persistent AIE changes in neurobiology and behavior. AIE increases adult alcohol drinking and preference, increases anxiety and reward seeking, and disrupts sleep and cognition, all risks for AUD. In addition, AIE induces changes in neuroimmune gene expression in neurons and glia that alter neurocircuitry and behavior. HMGB1 is a unique neuroimmune signal released from neurons and glia by ethanol that activates multiple proinflammatory receptors, including Toll-like receptors (TLRs), that spread proinflammatory gene induction. HMGB1 expression is increased by AIE in rat brain and in post-mortem human AUD brain, where it correlates with lifetime alcohol consumption. HMGB1 activation of TLR increase TLR expression. Human AUD brain and rat brain following AIE show increases in multiple TLRs. Brain regional differences in neurotransmitters and cell types impact ethanol responses and neuroimmune gene induction. Microglia are monocyte-like cells that provide trophic and synaptic functions, that ethanol proinflammatory signals sensitize or "prime" during repeated drinking cycles, impacting neurocircuitry. Neurocircuits are differently impacted dependent upon neuronal-glial signaling. Acetylcholine is an anti-inflammatory neurotransmitter. AIE increases HMGB1-TLR4 signaling in forebrain, reducing cholinergic neurons by silencing multiple cholinergic defining genes through upregulation of RE-1 silencing factor (REST), a transcription inhibitor known to regulate neuronal differentiation. HMGB1 REST induction reduces cholinergic neurons in basal forebrain and cholinergic innervation of hippocampus. Adult brain hippocampal neurogenesis is regulated by a neurogenic niche formed from multiple cells. *In vivo* AIE and *in vitro* studies find ethanol increases HMGB1-TLR4 signaling and other proinflammatory signaling as well as reducing trophic factors, NGF, and BDNF, coincident with loss of the cholinergic synapse marker vChAT. These changes in gene expression-transcriptomes result in reduced adult

neurogenesis. Excitingly, HMGB1 antagonists, anti-inflammatories, and epigenetic modifiers like histone deacetylase inhibitors restore trophic the neurogenesis. These findings suggest anti-inflammatory and epigenetic drugs should be considered for AUD therapy and may provide long-lasting reversal of psychopathology.

KEYWORDS

epigenetics, HMGB1, ethanol, alcohol, neurogenesis

Introduction

Individuals who begin drinking in their early teen years and during puberty have very high rates of adult alcohol problems and alcohol use disorder (AUD) [1]. However, causally relating early adolescent human adolescent drinking to high rates of adult AUD is confounded by multiple environmental and genetic factors that impact adolescent development, peer and family influences as well as emerging personality disorders and progressive increases in drinking trajectories into adulthood. Preclinical studies in rodents allow hypothesis testing on the impact of exposure to alcohol during adolescence that control for genetics and environment and can limit exposure to adolescent ages (i.e., without continuous alcohol exposure into adulthood). This design allows selective determination of the impact of ethanol on adolescent brain that persists into adulthood. The Neurobiology of Alcohol Drinking in Adulthood (NADIA) consortium designed the adolescent intermittent ethanol (AIE) exposure rat model to fit patterns of underage binge drinking. AIE involves alcohol exposure across what is equivalent to the teenage years in humans; in rats, this is approximately postnatal day 25 (P25) to P55, with females having puberty a bit before males, similar to humans. Following AIE, rats are allowed to mature to adulthood, usually P80–P90, equivalent to 30- to 40-year old humans, without any further alcohol exposure. The AIE model tests the hypothesis that AIE causes long-lasting persistent changes in adults that increase risks of adult alcohol problems and AUD. This model tests the impact of adolescent drinking while avoiding the human confounds, particularly genetic inheritance, that complicate understanding the strong relationship of adolescent drinking and later life AUD. In males, multiple AIE studies find increases in adult alcohol drinking [2–11]. AIE-induced adult rat drinking is increased after adolescent ethanol exposure in adults of both sexes, with females drinking more than males [12]. AIE ethanol self-administration and AIE ethanol vapor exposure also promote increases in adult operant responding for ethanol self-administration and reduce extinction [4, 13]. AIE ethanol exposure without adult ethanol exposure also increases adult anxiety and reduces behavioral flexibility and responses to acute alcohol, consistent with widespread changes in multiple cognitive-behavioral domains. Learning studies find AIE does not change young adult learning ability [4, 14–17], although complex operant tasks with rule changes and set-shifting show deficits [4] and as does some spatial–temporal object recognition [18]. Studies using the Morris water maze and the Barnes maze find initial learning is intact and not altered, but reversal learning, a measure of behavioral flexibility assessed by changing the goal location, reveals reversal deficits [18–23] due to perseveration and loss of executive function [24]. Adult rat responses in a probability discounting task that changes the ratio of arm pressing to food pellet reward find AIE increases risky choices [14, 25, 26] and enhances reward seeking in adulthood [27–30]. Another effect of AIE is heightened social anxiety in adulthood [31], particularly in males [32–34]. AIE also increases adult anxiety-like behavior using the elevated-plus maze [6, 35–37] or the light–dark box [5, 6, 37–39] or the marble-burying test [5], as well as the open-field test [21, 40]. These findings are consistent with the finding that AIE increases amygdala CRF [14]. Other reviews provide more details on the impact of AIE on persistent changes in adults behavior [24, 31, 41–44] as well as the review specifically on the role of sex in AIE [45]. In summary, adolescent alcohol exposure as modeled by AIE causes changes that increase risk factors for AUD that persist long after adolescence without additional alcohol exposure in adulthood. The mechanisms of these persistent AIE-induced changes could explain the link between age of drinking onset, lifetime AUD and alcohol-related problems.

The long-lasting changes in adult mood, cognition and reward following AIE are likely related to changes in neuronal networks that underlie self-reflection, attention and self-control mechanisms developing during adolescence. Understanding cellular mechanisms involved in adolescent maturation of brain neuronal networks and the impact of binge drinking provides important information for prevention efforts as well as targets for treatment and diagnosis. Both human [46–48] and preclinical studies [1, 24, 49] have found adolescent maturation alters brain physiology, networks, structure and function. Chronic adult binge drinking models, as well as the adolescent intermittent binge models, find changes in gene expression. Adolescent sensitivity to alcohol induced long-lasting changes in adults without further alcohol exposure in the NADIA AIE model [24, 49] in general are exaggerated responses occurring with less alcohol exposure than is needed in adult models. Proinflammatory neuroimmune genes are generally increased across models as well as in post-mortem

brain of individuals with AUD. Proinflammatory genes have been linked to AUD. Transcriptome studies find changes in large numbers of gene classes that consistently include neuroimmune and epigenetic modiflying genes. More recent transcriptome studies have established the importance of single cell studies that allow links to cell and network function. Emerging studies have identified neuroimmune triggered epigenetic modifications in microglia, astrocytes, and neurons that impact neuronal networks related to mood, cognition, and salience. Epigenetic changes are reversible, providing opportunities for new therapies. However, all cells respond to their surrounding cells in different limbic and cortical brain regions that likely contribute to variation. This review will touch on epigenetic mechanisms in response to neuroimmune signaling. It introduces a complex cytokine-like molecule, high-mobility group box 1 (HMGB1), as a key brain proinflammatory signal linked to alcohol-induced changes. Microglia are the innate immune cells of brain and are primed or sensitized by alcohol-linked HMGB1 proinflammatory signals. Microglial and astrocyte changes during cycles of alcohol exposure are proposed to interact with neurons through signals altering gene expression through complex mechanisms. AIE-induced changes in cholinergic (ChAT) basal forebrain neurons and hippocampal dentate gyrus neurogenesis are reviewed as examples of how neuronal networks linked to cholinergic arousal and new neuron formation undergo persistent adult cognitive deficits that can be restored through reversal of proinflammatory-epigenetic signaling.

Epigenetic mechanisms of AIE-induced AUD-like pathology

The mechanisms of AIE-induced changes in adult rat brain are linked to increases in neuroimmune gene expression across neurons, microglia, astrocytes and likely other brain cell types. Epigenetics has emerged as a mechanism of persistent, long-lasting changes in gene expression in response to environment, including enriched, stressful or trauma-induced changes [36, 49, 50]. Epigenetic gene regulation includes histone and DNA methylation and microRNA regulators of gene expression and cell phenotype reprogramming that have emerged as mechanisms of alcohol-induced changes in brain that are linked to proinflammatory signaling. Epigenetics shifts transcription through silencing or enhancing gene transcription [51–53]. Although neurons connect across brain regions, glial-neuronal signals regulate synapses and other interactions within each brain region. Studies of AIE find reduced trophic factor expression with increased proinflammatory gene expression which are persistent shifts in cellular transcriptomes lasting to adulthood, and which are reversible with anti-inflammatory or epigenetic modifying drugs. Binge alcohol exposure was first discovered to induce long-lasting changes in brain neuroimmune gene expression [54–57]. Chronic ethanol exposure of mice was discovered to increase brain Toll-Like receptors (TLR) and sensitize brain TLR4 [58] and TLR3 proinflammatory responses [59] that has emerged as mechanism regulating alcohol self-administration and preference in mice [60, 61], as well as following AIE in rats [24, 49]. Cycles of alcohol-induced innate immune memory processes increase TLR expression in brain, priming microglia and other cells and thereby increasing proinflammatory responses [62–64]. There are a large number of genes associated with the immune system, including adaptive immunity T and B cell lymphocytes, as well as innate immunity tissue-specific and blood monocytes [65]. Healthy brain does not have T or B lymphocytes or their associated antibodies and there are low levels of expression of innate immune genes with some being expressed transiently in neurons during development or initiation of synaptic plasticity. A large number of studies currently link ethanol drinking and preference to neuroimmune signaling using transcriptomic models [66–68], transgenic animal models [69, 70], post-mortem human brain immunohistochemistry and PCR [71–74], and AUD models [75]. In general, brain neuroimmune gene expression refers to genes associated with innate immune signaling, particularly proinflammatory cytokines such as TNFα, IL1β, and IL6. In healthy brain, these genes are expressed at very low levels but are sensitive to drugs, stress, and other environmental factors. A characteristic of proinflammatory innate immune signaling is that an initial signal from one cell activates multiple other cells and itself to increase expression of multiple proinflammatory cytokines, chemokines, and other genes. This results in many proinflammatory signaling molecules being involved in the lasting changes induced by chronic ethanol exposure. This review will focus on HMGB1, an endogenous protein expressed in all brain cells that has both nuclear and immune signaling proinflammatory functions [76]. High-mobility group (HMG) proteins were first identified as a class of nonhistone proteins that contribute to packaging DNA into chromosomes, with high-mobility group box 1 (HMGB1), emerging as an actively released protein with a key role in immune signaling [76, 77]. HMGB1 was discovered to bind neuroblasts and called amphoterin, but has emerged as an endogenous cytokine-like molecule that can activate multiple TLRs, previously discovered to respond to complex bacterial products in the immune system, but rarely studied in sterile brain. Examples of AIE-altered HMGB1 signaling and persistent changes in adult brain include adult hippocampal neurogenesis, microglial priming, and loss of basal forebrain cholinergic neurons. The mechanisms of AIE-induced persistent changes in HMGB1 and neuroimmune signaling are linked to lasting changes in adult perseveration, cognition, and AUD risk behaviors. (See Table 1).

HMGB1 expression in increased in post-mortem human AUD hippocampus as well as ethanol-exposed rats and mice and AIE-treated adult rats [71, 100]. AIE also induces subtle but persistent increases in hippocampal expression of the

TABLE 1 Select articles on HMGB1, adolescence, and alcohol.

Preclinical and clinical alcohol exposure effects on HMGB1 primary literature				
Species	**Exposure**	**Assessment**	**Results**	**Reference**
Rat (Wistar)	AIE	Prefrontal cortex (PL, IL)	↑ HMGB1 (IHC, mRNA), also TLR4, TLR3 (mRNA) in P56 and P80 adult rats. HMGB1 colocalizes with neurons (NeuN). AIE rats also exhibit reversal learning deficits.	[23]
Human	AUD	Orbitofrontal Cortex	↑ HMGB1 correlated with earlier age of drinking onset (IHC), also ↑ RAGE	[74]
Rat (Wistar)	AIE	Orbitofrontal Cortex	↑ HMGB1 (IHC) and ↑RAGE	[74]
Rat (Sprague)	CE (7% liquid diet, 15 days), or CIE (7% liquid diet intermittent)	Cortex (whole brain)	↑ HMGB1 (mRNA) during CE and CIE withdrawal but not intoxication; also increased TLR4 (mRNA) but no change in MyD88 (mRNA) or NFκB (mRNA)[a] ↑ HMGB1 (mRNA) during CE and CIE withdrawal blocked by CRF1 antagonist (CP154,526: 10 mg/kg) and ethyl pyruvate (75 mg/kg) but not the HMGB1 antagonist glycyrrhizin	[78]
Human	AUD	Orbitofrontal Cortex	↑ HMGB1 correlates with TLR and age of drinking onset	[72]
Rat (Wistar)	0 → 100 mM EtOH	hippocampal- entorhinal cortex organotypic slice culture	↑ HMGB1 (mRNA), ↑ HMGB1 released into media (ELISA)	[72]
Rat (Wistar)	0 → 100 mM EtOH	hippocampal- entorhinal cortex organotypic slice culture	Ethanol dose dependently ↑ HMGB1 (mRNA) and ↑ HMGB1 released into media (ELISA). Acetyl-HMGB1 is released; HDAC inhibitors also increase acetyl-HMGB1 release into media	[79]
Rat (Wistar)	AIE	Hippocampus	↑ HMGB1 (mRNA)	[80]
Human	AUD	Hippocampus	↑ HMGB1 (WB) ↑ HMGB1/1L-1β complexes (WB)	[81]
Mouse	Acute 6 g/kg i.g.	Whole brain Cortex Plasma Liver	↑ HMGB1 (ELISA, IHC, WB) ↑ HMGB1/1L-1β complexes (Western blot, IHC) ↑ HMGB1 (ELISA) ↑ HMGB1 (WB)	[81]
Human	AUD	Hippocampus	↑ HMGB1 in Human AUD Hippocampus (ELISA)	[82]
Rat	25–100 mM ethanol (48 h)	hippocampal- entorhinal cortex organotypic slice culture	↑ MV-HMGB1 (ELISA) and miRNA Let7 ↑ HMGB1/Let7 complexes in MV (ELISA)	[82]
Rat (Wistar)	AIE	Hippocampus	↑ HMGB1, TLR4, TNFα, IkBα (mRNA) and loss of neurogenesis (DCX, IHC) [a]Prevented with concurrent voluntary exercise or indomethacin	[83]
Human (young adult) ♀ ♂	Binge Drinkers	Serum	↑ HMGB1 (ELISA) in female but not male subjects following acute binge alcohol	[84]
Rat (Wistar)	AIE	Hippocampus	↑ HMGB1 (IHC), ↑ RAGE, ↑ TNFRSF25, cleaved caspase-3, pNFκB-p65 [a]HMGB1 changes not reversed with donepezil; other proinflammatory markers reversed by donepezil	[85]
Mouse/Human cell line	100 mM EtOH (24 h)	BV2, SH-SY5Y BV2+ SH-SY5Y co-culture	24 h EtOH did not impact HMGB1 (mRNA) in BV2, SH-SY5Y or co-culture 24 h EtOH ↑HMGB1 release into media in BV2 and SH-SY5Y cultures but not in co-cultured BV2+SH-SY5Y preps. IL-4 and	[86]

(Continued on following page)

TABLE 1 (*Continued*) Select articles on HMGB1, adolescence, and alcohol.

Preclinical and clinical alcohol exposure effects on HMGB1 primary literature				
Species	**Exposure**	**Assessment**	**Results**	**Reference**
			IL13 mRNA increased in co-culture EtOH EtOH ↑ TLR4 (mRNA)in co-culture BV2/SH-SY5Y, but co-culture attenuated EtOH TLR3/TLR7 (mRNA) and iNOS (mRNA)	
Human (AUD)	AUD	Orbitofrontal Cortex	AUD increases multiple TLR and NFκB genes that correlate with increased expression of HMGB1	[73]
Rat (Wistar)	AIE	Basal Forebrain	↑ HMGB1 (IHC) with ↑TLR4, ↑ pNFκB p65, and ↑ RAGE as well as ↑ H3K9me2 and decreased ChAT by AIE[a] Galantamine prevented/reversed AIE-induced changes in adulthood	[87]
Rat (Wistar)	AIE	Dentate gyrus of the hippocampus	↑ HMGB1 (IHC) and other proinflammatory markers including CCL2, COX2 and cleaved Caspase-3 while decreasing neurogenesis (DCX) [a]galantamine prevented/reversed	[88]
Human ♀ ♂	AUD, ALD	Serum	↑ HMGB1 in ALD relative to AUD (ELISA); predicts mortality in AUD.	[89]
Rat (Wistar)	*In vivo*: AIE *Ex vivo*: dsHMGB1 and rHMGB1, 100 mM EtOH for 4 days	*In vivo*: Basal Forebrain *Ex vivo*: BFCN organotypic slice culture	*In vivo*: ↑ HMGB1 (mRNA) *Ex vivo*: dsHMGB1 and rHMGB1 both reduce ChAT. Ethanol releases HMGB1 into media. REST and G9a induction lead to ChAT gene silencing. Loss of ChAT blocked by HMGB1 antagonist glycyrrhizin	[90]
Rat (Wistar) ♀ ♂	AIE	Dentate gyrus of the hippocampus	↑ HMGB1 (IHC) [a]Indomethacin reversed AIE-induced loss of neurogenesis and cholinergic markers and reduced HMGB1 (IHC)	[91]
Other HMGB1-RELATED primary literature				
Species	**Exposure**	**Assessment**	**Results**	**References**
Rat (unspecified)	0–5 mM Glutamate; 0–100 μM NMDA	hippocampal- entorhinal cortex organotypic slice culture	Glutamate dose-dependently ↑ HMGB1 release into media parallel to ↑ cell death (exclusion dye propidium iodide). NMDA similarly dose-dependently ↑ HMGB1 release into media parallel to ↑ cell death (exclusion dye propidium iodide).	[92]
Vglut2-Cre/ChR2-eYFP mice	ChR2 stimulated	*In vivo*: DRG *Ex vivo*: DRG neuronal culture	↑ HMGB1 cytoplasmic translocation (IHC) ↑ HMGB1 release (WB/ELISA)	[93]
Syn-Cre/HMGB1fl/flMice	Neuronal HMGB1 ablation	DRG	Neuronal HMGB1 ablation reduced hyperalgesia following sciatic nerve injury and attenuated proinflammatory cytokine and chemokine responses (ELISA: TNFα, CXCL1, IL18)	[93]
Rat (Wistar)	*In vivo*: LPS (1 mg/kg, i.p.); *Ex vivo*: LPS (100 ng/mL), dsHMGB1	Basal forebrain (*in vivo*); BFCN organotypic slice culture (*ex vivo*)	dsHMGB1 and LPS trigger TLR4 induction of REST and G9a gene silencing to cholinergic transcriptome.	[94]
CD-1 mice	Radioactive labeled HMGB1	Whole brain Serum	HMGB1 is transported across the BBB in both directions. LPS exposure ↑HMGB1 transport in part by disrupting	[95]

(Continued on following page)

TABLE 1 (*Continued*) Select articles on HMGB1, adolescence, and alcohol.

Other HMGB1-RELATED primary literature				
Species	**Exposure**	**Assessment**	**Results**	**References**
			the BBB and in part through a transport mechanism.	
Swiss albino or transgenic Thy1-ChR2- YFP and hGFAP-GFP adult mice	optogenetic stimulation or pinprick for cortical spreading depolarization	Cortex	↑ HMGB1 nuclear translocation (IHC) and ↑ HMGB1 extracellular vesicles with some indication of astrocyte-HMGB1 but not microglial-HMGB1 interactions	[96]

HMGB1-Related review articles	
Findings	**Reference**
This review covers the rapid release of HMGB1 from neurons during a seizure, increasing astrocyte and microglial IL-1β/HMGB1 synthesis and release. Long lasting decreases in seizure threshold are linked to persistent increases in these signals.	[97]
Proposed the hypothesis that neuroimmune signaling contributes to the neurobiology of alcohol and substance use disorders.	[55]
The review covers evidence supporting drug induced increases in TLR in brain, particularly microglia, that respond to HMGB1 and microRNAs (miRNAs). Studies supporting ethanol enhanced TLR innate immune signaling changes gene transcription through epigenetic mech anisms alternating synapses and neuronal networks. Addiction involves progressive stages of drug binge intoxication and withdrawal that are linked to progressive increases in TLR signaling.	[71]
This review discusses HMGB1 oxidation-reduction and changes activities through multiple cell surface receptors. Also, this review discusses recent discoveries indicating that HMGB1 released from neurons mediates inflammation via the TLR4 receptor system.	[98]
The studies reviewed support roles for neuroimmune signaling as well as epigenetic reprogramming of neurons and glia, which create a vulnerable neuro- environment. Some of these changes are reversible, giving hope for future treatments to prevent many of the long-term consequences of adolescent alcohol abuse.	[99]
AIE increases adult alcohol drinking, risky decision-making, reward-seeking, and anxiety as well as reducing executive function that increase risks for AUD. AIE causes persistent increases in adult brain neuroimmune signaling high-mobility group box 1 (HMGB1), TLR, RAGE and other innate immune genes. These genes are also increased in human AUD brain. HMGB1 release by ethanol, both free and within extracellular vesicles shifts transcription and cellular phenotype. For example, RE-1 silencing transcript blunts cholinergic gene expression, shifting neuronal phenotype. Inhibition of HMGB1 neuroimmune signaling, histone methylation enzymes, and galantamine, the cholinesterase inhibitor, both prevent and reverse AIE pathology. These findings provide new targets that may reverse AUD neuropathology as well as other brain diseases linked to neuroimmune signaling.	[1]
This is a review of HMGB1 immune cell functions including promoting DNA damage repair in the nucleus, sensing nucleic acids and inducing innate immune responses and autophagy and stimulating immunoreceptors. Signaling, cellular functions and clinical relevance of HMGB1 in various diseases are discussed.	[77]

[a]AIE, adolescent intermittent ethanol; AUD, alcohol use disorder; ALD, Alcohol-related Liver Disease; BBB, blood brain barrier; CCL2, c-c motif ligand 2; COX2, cyclooxygenase-2; DCX, doublecortin; DRG, dorsal root ganglion; dsHMGB1, disulfide high mobility group box 1; ELISA, enzyme-linked immunosorbent assay; EtOH, ethanol; IHC, immunohistochemistry; IL, infralimbic; NeuN, neuronal nuclear protein; LPS, lipopolysaccharide; MV, microvesicle; pNFκB, phosphorylated nuclear factor kappa-light chain enhancer of activated B cells; PL, prelimbic; RAGE, receptor for advanced glycation end products; TNFRSF25, tumor necrosis factor receptor superfamily 25; WB, western blot.

proinflammatory signaling factors chemokine C-C motif ligand 2 (CCL2), cytokines TNFα and IL1β, and cyclooxygenase-2 as well as expression of innate immune signaling Toll-like receptors (i.e., TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR8) [73] and the receptor for advanced glycation end products (RAGE) and other proinflammatory signaling cytokines signal through feed-forward amplification innate immune receptors and their activating ligands. Interestingly, HMGB1 is actively released following acetylation [101] and we found ethanol, histone deacetylase inhibitors, and glutamate increase hippocampal brain slice culture release of HMGB1 into the media [79]. Studies in culture find ethanol releases HMGB1 from neurons [79] and microglia [82]. HMGB1 can form monomers as well as dimers and heteromeric complexes that function as a pan-

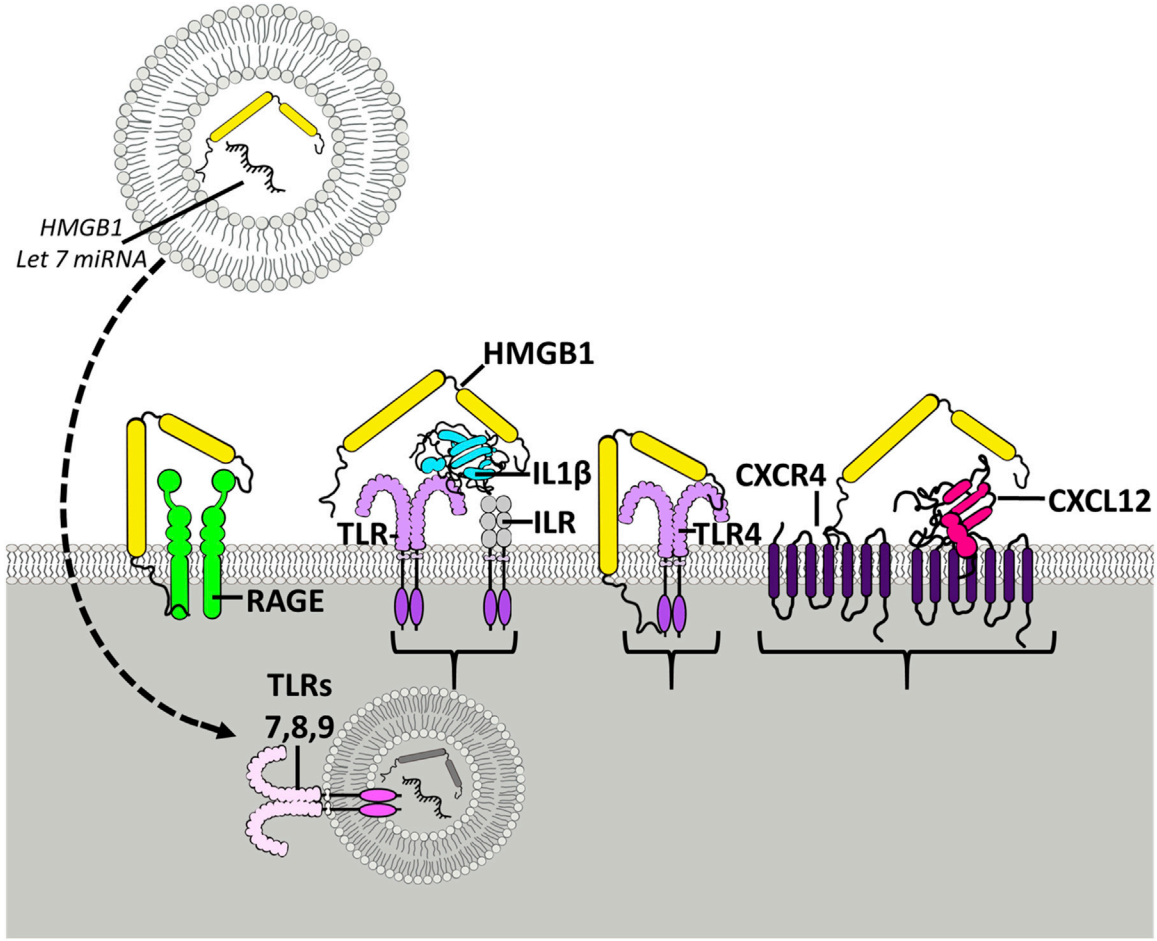

FIGURE 1
HMGB1 activates multiple receptors spreading neuroimmune signaling. Shown is the HMGB1 (yellow) molecule with two yellow Box sections, known as Box A and Box B, that bind to different molecules and receptors. The Box sections aggregate-stabilize (dimerize) receptor subunits, increasing activation. HMGB1 can stimulate TLR4 receptors directly and as heteromers with other TLR agonists. TLR receptors are members of the TLR-IL1 receptor family that are activated by agonist dimerization. Receptors are drawn as active dimers with HMGB1 bridging dimers, the hypothetical mechanism of HMGB1 potentiating receptor responses. Shown is HMGB1 alone stimulating TLR4 and RAGE receptors. HMGB1 is known as a "sticky" protein binding to lipids, RNA, DNA, and chemokine proteins. On the right is shown HMGB1-CXCL12 heteromers bridging G-protein receptors. HMGB1 has been found to enhance the potency of CXCL12 at CXCR4 receptors, G-protein-linked chemokine receptors [105] activated by dimerization. Another example involves IL1β-ILR receptors (TLR/ILR receptor family) which act through HMGB1/IL1β heteromers, increasing potency at the ILR over that of IL1β alone [100]. Similarly, studies find microglial activation releases HMGB1 as a heteromer in microvesicles with microRNA let-7, an endogenous TLR7 agonist that when combined with HMGB1, that is able to activate TLR7 in adjacent neurons [106]. HMGB1 complexes can activate essentially all TLRs [102], contributing to HMGB1 as a proinflammatory signal. HMGB1 has broad neuroimmune stimulating activity crossing multiple innate immune receptors.

proinflammatory amplifying factor. HMGB1 heteromeric complexes form with cytokines, extracellular DNA, RNA, and damage-associated molecular pattern (DAMP) molecules [93]. HMGB1 heterocomplexes are able to activate TLRs, making TLRs an important proinflammatory signal [102]. For example, TLR7 is activated by RNA, including endogenous miRNA let7 and HMGB1-let7 dimers, which are both potent agonists. Interestingly, ethanol releases HMGB1-let7 dimers in extracellular vesicles (EVs) from microglia, triggering TLR7-mediated pathology [82]. Multiple studies suggest TLR7 is linked to increases in preclinical alcohol drinking and preference [103, 104]. The ability of HMGB1 to activate and amplify proinflammatory signals positions it as a key target to block proinflammatory gene induction (Figure 1). Alcohol and substance abuse disorders involve a progression of increased drug taking with activation of reward centers, followed by mood dysfunction and limbic involvement with increasing involvement of prefrontal and other cortical dysfunction [107] that could represent progressive increases in HMGB1 and/or other neuroimmune signals. It is nearly impossible to measure all

proinflammatory signals, with most studies focusing on TNF, IL1B, IL6 or CCL2. The classical acute phase innate immune systemic blood response to infection involves these and other proinflammatory cytokines and chemokines, consistent with all being representative of neuroinflammation. This is an oversimplification since neurons, astrocytes, microglia and other brain cells respond to an initial proinflammatory response with different cytokines that vary dependent upon the surrounding mileu and brain region that alters the spread of proinflammatory signaling. One example linking HMGB1, proinflammatory signaling and ethanol pathology is sensitization to pain. Pain as assessed by tactile allodynia increases following nerve injury due to changes in neurons and the local microglia [108]. Spinal cord microglia are activated contributing to pain sensitization [108]. In models of pain, nociception sensory neurons have increased HMGB1 and increases in HMGB1 release with increased pain. Antibodies to HMGB1 have been block neuropathic pain [109]. Neuronal activation using optogenetic mechanisms increases release of HMGB1 [93] that can activate microglia. Further, silencing of HMGB1 protects against both nerve injury and proinflammatory pain models [93]. These findings are consistent with ethanol induction and neuronal release of HMGB1 contributing to local microglial sensitization that persists and amplifies proinflammatory responses that impact neurocircuitry. Studies finding HMGB1 release from multiple brain cells is consistent with initiating proinflammatory signaling, although the details on reward, affect, and cognitive neurocircuitry is not known. Understanding the mechanisms of progressive increases in brain HMGB1-TLR proinflammatory signaling across brain regions, neurocircuits, and components of psychopathology will benefit both prevention and treatment efforts.

Microglia, HMGB1, and alcohol

Microglia are brain-specific monocyte-like cells that are long-lived but can also divide from endogenous progenitors throughout the lifespan [110]. Microglia within each brain region is relatively stable and if altered, microglia proliferate to return to the "homeostatic" density, suggesting local regulatory microglial niche mechanisms [111]. Microglia are suggested to control the escalation of drinking in mouse models of alcohol dependence [112], consistent with escalation of drinking being linked to amplification of HMGB1 -proinflammatory signaling increases with repeated exposure [71, 100]. During striatal development microglia regulate dopamine receptors, with male sex-specific microglial elimination of striatal synaptic dopamine D1 receptors through microglial-transcytosis, i.e., synaptic receptor specific phagocytosis, that precedes the development of male specific adolescent play behaviors [113]. In transgenic mice with depleted numbers of microglia, there is reduced adolescent synaptic pruning, resulting in more synapses but reduced cortical function [114]. Interestingly, cortical microglial gene expression correlates with cortical thickness during childhood and early adolescence [115], and cortical thickness is linked to development of adult characteristics [116, 117]. Though microglia are critical for neurodevelopment during adolescence, in general, little is known about microglia and their role in specific neurocircuitry. What is known is that microglia have multiple phenotypes that are regulated through epigenetic mechanisms [118] and adolescent ethanol exposure causes long lasting sensitization and other alterations in brain microglia [49, 119, 120].

Microglia contribute to acute alcohol responses [82, 121, 122] and become sensitized to proinflammatory signals like HMGB1. Sensitization or priming of microglia by stressors or TLR agonists persists [123, 124], and priming increases expression of complement pathways that regulate synaptic plasticity [125]. For example, AIE adolescent binge ethanol exposure followed by 45 days of abstinence increases adut restraint stress Cd11b+ microglia activation in frontal cortex and amygdala [121]. Adolescent stress also increases adult microglia responses to lipopolysaccharide (LPS) [126], consistent with studies finding ethanol sensitizes to LPS [127]. Another adolescent binge ethanol exposure study found disruption of novel object learning and hippocampal long-term synaptic depression are blocked by microglial inhibitor minocycline and TLR4 antagonist TAK-242, as well as the anti-inflammatory drug indomethacin [128]. Another AIE study found increased pain sensitivity in adults that was alleviated by minocycline [129]. These studies support AIE priming of microglia, although stress can also prime microglia; adolescent alcohol and stress sensitize and synergize to increase proinflammatory responses in some brain regions but not others [121]. Recent studies report blood monocytes of individuals with AUD are primed to TLR4 proinflammatory responses [130]. These studies suggest microglial priming contributes to increases in alcohol drinking and AUD psychopathology.

Immune signaling and acetylcholine

Although in general microglia and proinflammatory signaling are linked to the mechanisms that underlie the development of AUD, proinflammatory responses are complex. One example is the pain circuit, which has both central and peripheral components and the anti-inflammatory actions of acetylcholine [131, 132]. Both adult and adolescent AIE are found to sensitize pain responses [133, 134]. HMGB1, microglia and proinflammatory signals are linked to pain sensitivity. Acetylcholine inhibits microglia and the vagus nerve sends projections to the organs that inhibit proinflammatory responses with acetylcholine [135–137]. The inflammatory reflex signals are anti-inflammatory nerve signals

that stimulate a subset of immune cells to secrete acetylcholine, which interacts with alpha 7 nicotinic acetylcholine receptors to inhibit proinflammatory mediators [138, 139]. Thus, acetylcholine is known to reduce proinflammatory signaling and brain regions with high levels of acetylcholine will show less proinflammatory induction by ethanol and other insults than brain regions without any cholinergic anti-inflammatory signaling.

HMGB1 and epigenetic regulation of forebrain cholinergic neurons

Forebrain cholinergic neurons projection to multiple cortical and limbic brain regions, including the cortex, hippocampus, and amygdala. Cholinergic neurons modulate arousal, cognitive and emotion [140, 141]. AIE reduces expression neuronal choline acetyltransferase (ChAT) in the medial basal forebrain and shrinks remaining ChAT + IR cholinergic neurons size [18, 20, 22, 40, 142–145]. The vesicular ACh transporter (VAChT), and the high- and low-affinity nerve growth factor receptors TrkA and NGFR, all cholinergic neuron markers are also decreased [22, 83, 142]. The AIE-induced loss of basal forebrain cholinergic neurons is accompanied by diminished ACh prefrontal cortical efflux during maze performance [144]. The forebrain ChAT+ cell loss is selective, since parvalbumin GABAergic neurons in the basal forebrain are not reduced by AIE [20]. AIE deficits in reversal learning are linked to the ChAt loss by anti-inflammatory indomethacin, exercise, and galantamine treatments during AIE that prevent the loss of ChAT+ neurons and cognitive deficits [22, 142, 145]. The TLR4 agonist lipopolysaccharide (LPS) activates brain proinflammatory signaling and treatment during adolescence mimics the AIE-induced loss of ChAT [40, 145]. AIE induces forebrain TLR4 and RAGE receptors, HMGB1, and the nuclear transcription factor pNFkB p65 proinflammatory signaling transcription factor [40, 145]. Rat voluntary wheel running exercise, and indomethacin prevent AIE induction of HMGB1-TLR4/RAGE-pNFκB p65+IR within ChAT + IR neurons, their loss and shrinkage [145]. Historically, loss of terminally differentiated ChAT+ neurons was interpreted as cell death and considered irreversible. However, emerging studies find brain proinflammatory signals are induced by epigenetic changes in microglia and neurons that are reversible. Studies found that reduced ChAT+ neurons, and shrunken ChAT+ neurons could be restored after AIE treatment. Exercise running wheels reversed AIE increased forebrain HMGB1-TLR4 and RAGE-as well as the loss of ChAT+, TrkA+, and NGFR+ cholinergic neurons and somal shrinkage. There were no changes in total NeuN+ neuron numbers and no neurogenesis, suggesting neurons did not die but only lost the cholinergic phenotype, allowing restoration [22, 142]. These findings were extended with anti-inflammatory treatments indomethacin and galantamine, which acts through enhanced acetylcholine as an anti-inflammatory treatment. More recent studies have discovered transcriptional repressor RE1-silencing transcript (REST; also known as neuron-restrictive silencer factor [NRSF]) [146, 147] regulate cholinergic gene expression [147] and is known to bind methyltransferase G9a, increasing histone H3K9 dimethylation that represses gene expression [148, 149]. HMGB1 signaling was discovered to increase REST-G9a silencing of multiple genes that define a cholinergic neuron, and that reversal of REST-G9a silencing restored the cholinergic neurons [90]. The findings that adolescent binge ethanol exposure and neuroimmune induction have epigenetic components that are reversible create promise for new AUD therapies [1, 52, 150–153].

The hippocampal neurogenic niche and alcohol

The hippocampal dentate gyrus subgranular zone is a unique brain region where new neurons are formed well into adulthood. New neurons form from proliferating progenitors that become mature neurons which functionally integrate into neurocircuitry in adulthood [154, 155]. The local environment is a “neurogenic niche” regulating the birth, differentiation, and functional integration of hippocampal newborn neurons. The niche is sensitive to disruptions that alter trophic support due to increased proinflammatory signaling [156]. Ethanol exposure reduces hippocampal neurogenesis due in part to changes in the neurogenic niche [156]. Models of AUD binge drinking in adults find ethanol inhibits hippocampal neurogenesis transiently that recovers during abstinence [54, 119, 157]; however, adolescents which have about 4-fold more neurogenesis than adults [158, 159] show a persistent loss following AIE adolescent AIE exposure, far greater than that with identical adult alcohol treatment [160]. Further, the AIE-induced loss of neurogenesis persists for months, likely for life [80]. AIE inhibition of hippocampal neurogenesis following AIE is associated with adult reversal learning impairments, increased perseveration and/or loss of cognitive flexibility, which persist at least to middle age in rodents [80, 161]. The niche is disrupted by AIE. AIE increases hippocampal proinflammatory HMGB1, COX2 and other proinflammatory genes [83, 85, 88]. And reduces expression of trophic factors, specifically BDNF [37]. Interestingly, the AIE-induced loss of adult neurogenesis is reversible. Exercise and anti-inflammatory drugs (e.g., indomethacin, donepezil, and galantamine), as well as the epigenetic histone deacetylase inhibitor, trichostatin A (TSA) prevent and/or restore the AIE-induced loss of neurogenesis as well as the lasting perseveration and loss of behavioral flexibility [37, 83, 85, 88]. AIE increases HMGB1 and other proinflammatory genes [83] and decreases in the trophic factor BDNF [37], suggesting that AIE disrupts the

neurogenic niche through a transcription shift increasing proinflammatory genes while reducing trophic gene expression through epigenetic gene silencing and enhancer mechanisms. The proinflammatory HMGB1 reduced trophic expression changes in gene expression and the loss of neurogenesis that are reversed by anti-inflammatory treatments like indomethacin [91] as well as the histone deacetylase inhibitor TSA [37] supports the hypothesis of epigenetic shifts driven by proinflammatory signals that reduce neurogenesis. More specifically, indomethacin, the non-steroidal anti-inflammatory drug and the cholinesterase inhibitors galantamine and donepezil reverse AIE-induced loss of neurogenesis and increases in hippocampal HMGB1 [85, 88]. TSA, a histone deacetylase inhibitor that reverses epigenetic proinflammatory activation in microglia as well as other cells, restores hippocampal BDNF and AIE-neurogenesis [37]. TSA also reverses AIE-induced changes in amygdalar histone acetylation, reverses AIE adult anxiety, and reverses AIE induced increases in ethanol self-administration [6]. Restoration of neurogenesis also restores cognitive flexibility deficits during reversal learning on the Morris water maze [83]. The changes in the niche are complex. For example, AIE reduces cholinergic innervation of the niche, and anti-inflammatory treatment restores cholinergic innervation with the return of neurogenesis (for review see [156]. Although the folklore of Alcoholics Anonymous is "Once an alcoholic, always an alcoholic," thereby arguing AUD is a chronic disease, the findings that the AIE-induced AUD-like pathology is reversible provide a foundation for AUD cures. Understanding the brain region-specific mechanisms of AIE persistent pathology could lead to new and novel therapies for AUD.

Discussion and summary

Adolescent drinking is known to result in high rates of adult alcohol problems and lifelong AUD. To tests hypotheses on the lasting impact of adolescent drinking, the AIE adolescent binge drinking model assesses behavior and neurobiological mechanisms after several weeks of abstinent maturation to adulthood. AIE increases alcohol drinking and preference, anxiety, reduces adult social interaction, increases pain sensitivity and other hyperkalifia-like symptoms, as well as altering decision making while increasing perseveration and reversal learning deficits. Environment and access to alcohol contribute to the development of AUD; increased alcohol drinking, hyperkalifia, and reduced executive function following AIE are consistent with increasing risks of developing AUD in adulthood. The high rates of lifetime AUD following adolescent binge drinking have been suggested to be due to a lower adolescent intoxication response to alcohol, resulting in greater and sometimes extreme binge drinking that insults the developing adolescent brain. Adolescent brain is more sensitive to acute binge alcohol exposure [162, 163]. Although brain cellular damage is increased in models of adolescent binge drinking [162] and human AUD brain is generally smaller than moderate drinking controls, AIE studies indicate that the persistent, long-lasting impact of adolescent binge drinking is far broader than cellular damage due to changes in cells and neurocircuits induced by alcohol that persist long after alcohol exposure.

The discovery that neuroimmune signaling is linked to alcohol use disorder and alcohol drinking has emerged during the past decade. This review focuses on HMGB1, a molecule that is expressed in all brain cells, is localized in the nucleus, and that is actively released from cells following acetylation by histone acetylases. Ethanol increases neuronal histone acetylation in brain [164], and in brain slice cultures, ethanol releases acetylated HMGB1 into the media. HMGB1-histochemistry shows increases in neuronal cytoplasm consistent with active neuronal release [165]. Although poorly understood and confounded by cell death-triggered release, ethanol likely releases HMGB1 from multiple brain cell types, which sensitizes microglia and astrocytes to progressive increases in a large number of proinflammatory genes. Dependent upon brain region, each acute binge drinking episode can amplify and spread proinflammatory signaling. Proinflammatory signaling is associated with sickness behaviors that fit well with the negative emotional, hyperkatifeia [166, 167] affect stages of the development of AUD. Interestingly, ethanol acutely blocks monocyte responses that change within hours, increasing proinflammatory gene expression; that is, alcohol withdrawal coincides with increases proinflammatory cytokines [54, 121]. Binge drinking and associated acute withdrawals are proposed to prime microglia, and likely astrocytes, sensitizing and amplifying proinflammatory genes. Repeated withdrawals drive hyperkatifeia responses that promote further drinking that progressively involves altered neurocircuitry across reward, negative affect-hyperkatifeia linked and finally executive control dysfunction, leading to perseverative compulsive craving. Although it is poorly understood how various neurocircuits become progressively involved in the development of AUD, some insight is provided by studies of HMGB1 and hippocampal seizures. Hippocampal seizures induce persistent increases in HMGB1-TLR4 and IL1β which increases excitability, reducing seizure thresholds, i.e., sensitizing to future seizures, due to increases in HMGB1 and IL1β [97]. Similarly, cycles of chronic intermittent ethanol that progressively increase anxiety and negative effect are linked to HMGB1 amplification of amygdala TLR4 and changes in CRF with multiple withdrawals that are blocked by CRF1A and HMGB1 antagonists [78]. Although adolescents are proposed to be more sensitive to the impact of repeated exposure to ethanol than adults, HMGB1 is induced by ethanol at all ages, which could contribute to epigenetic

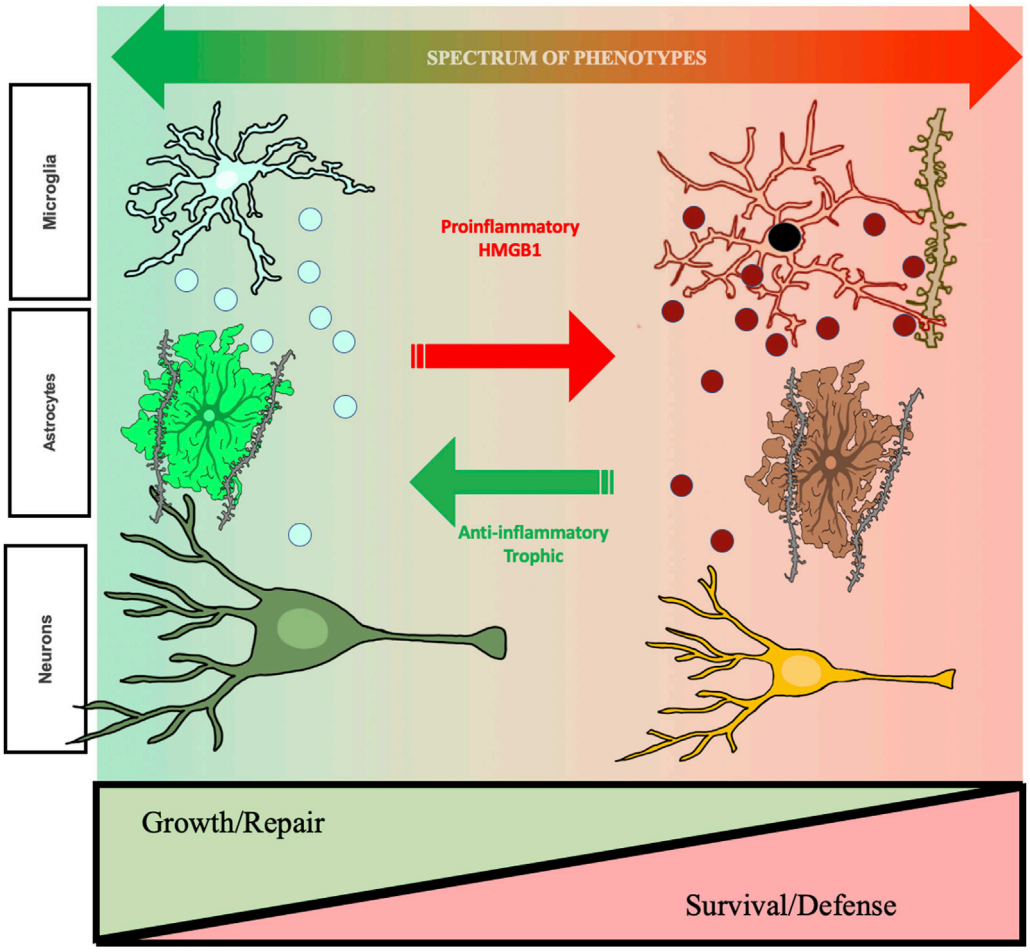

FIGURE 2
Hypothetic mechanism of ethanol-induced changes in cellular phenotypes related to changes in aud behavioral phenotypes. The studies reviewed find repeated cycles of binge drinking prime microglia, increase proinflammatory HMGB1, and alter brain and behavior that increases risk for AUD. Shown are microglia, astrocytes, and neurons. Left side green healthy microglia are trophic and release factors supporting a local growth repair milieu with other brain cell types including astrocytes, that help maintain synapses, and neurons. Chronic ethanol exposure "primes" microglia, that over repeated cycles converting them to a proinflammatory phenotype with increases in expression of CD68, a dark microglial phagocytic protein stain, and secretion of TNFα that persist for long periods and may impact synapse phagocytosis. Chronic studies of adolescent AIE find astrocytes also undergo a phenotype change, with alterations in GFAP and soma as well as reduced astrocyte-excitatory synapse PSD95 contacts. These long-lasting changes in astrocytes may represent a phenotype shift. Under healthy physiological conditions, astrocytes close synaptic contact with glutamatergic terminals where they regulate the synaptic environment and mediate glutamate homeostasis. This can be visualized using a combination of excitatory synaptic markers, glial-fibrillary actin protein (GFAP), and virus mediated astrocyte labeling with GFP. AIE causes hippocampal astrocytes to increase GFAP immunoreactivity in both sexes, indicating a shift towards a reactive phenotype, coupled with retractions of astrocytic processes from contact with excitatory synapses. These changes have critical functional implications for the role of astrocytes on mediating glutamate transmission, innate immune activation, and excitotoxicity. As described in the text, cholinergic neurons also change phenotype, some neurons lose the cholinergic phenotype and others show shrinkage of soma and loss of cholinergic markers in frontal cortex and hippocampal projections. Some neurons are no longer cholinergic, and remaining neurons have small soma suggest neuronal phenotype changes. These changes are associated with cognitive deficits, suggesting altered neurocircuitry. Evidence supports epigenetic mechanisms persistently shift cellular phenotype, but are reversible by exercise and other anti-inflammatory treatments. Reversal of phenotype changes also reverses behavioral deficits. Studies in the text support proinflammatory activation as altering cellular phenotypes from healthy growth repair to survival phenotypes that associate with ethanol induced changes in cognition and reward seeking, behavioral phenotypes with increased risks for AUD. Taken together, these results support that ethanol-induced changes in neuroimmune signaling mediate changes neurocircuitry that increase risks for AUD, but that are reversible.

mechanisms altering microglial phenotypes that impact synapses and neurocircuits. Adolescent intermittent ethanol is known to induce anxiety and increase alcohol drinking through reversible epigenetic mechanisms that alter synaptic proteins [52, 151]. These findings are consistent with multiple studies finding neuroimmune activation promotes alcohol drinking which

induces additional glial activation and epigenetic shifts in phenotypes across brain regions and cells (Figure 2).

Cholinergic neurons and hippocampal neuronal stem cells are two cell types presented as examples of how HMGB1-TLR proinflammatory signaling can directly alter neurocircuitry. AIE-induced loss of both ChAT+ neurons and hippocampal neurogenesis are prevented by indomethacin, an anti-inflammatory drug, and anti-cholinesterases, which increase acetylcholine and inhibit inflammation. Anti-inflammatory drugs are under investigation for treatment of AUD [63]. HMGB1-TLR4 signaling causes partial cholinergic neurons loss with remaining neurons shrunken due to induction of epigenetic silencing mechanisms. AIE-induced loss of ChAT+ neurons persists long into adulthood, likely for life, unless inhibited by anti-inflammatory or epigenetic drugs. This represents a phenotypic change in cholinergic phenotype. Although it is not clear, forebrain cholinergic-GABAergic neurons are common and lost ChAT+ neurons may remain GABAergic, altering target region circuitry. Cholinergic neurons respond to NGF, which is an important trophic factor reduced by AIE in target regions that could contribute to the reduced cholinergic transcriptome. This is consistent with proinflammatory-trophic transcription shifts in reducing cholinergic cellular phenotype. Interestingly, *in vivo* and *in vitro* reversal by anti-inflammatory, TLR4 antagonist or drugs that block epigenetic changes supports persistent proinflammatory signaling as maintaining epigenetic shifts in cholinergic phenotype. The reversal of epigenetic changes offers great promise for treatment of the chronic disease AUD. Changes in hippocampal neurogenesis similarly suggest proinflammatory increases and reduced BDNF trophic support alter the neurogenic niche, reducing adult hippocampal neurogenesis. Multiple cell types regulate the neurogenic niche and the proposed proinflammatory-induced shifts in phenotype are proposed for multiple cell types (Figure 2). Additional studies of how proinflammatory changes in cell transcriptomes and phenotypes contribute to progression to AUD across various brain regions will provide opportunities to develop improved treatments the have the promise of a cure through anti-inflammatory and epigenetic reversal of transcriptome shifting brain cell phenotypes.

Author contributions

All authors contributed to the research, figures and preparation. FC primarily wrote the review and VM prepared the figures with input from FC. RV edited. The authors thank Jennie Vaughn for excellent editorial and bibliography support.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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