IS EARLY ONSET OF ALCOHOL USE ASSOCIATED WITH LATER ALCOHOL USE?

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PUBLISHED IN: Frontiers in Behavioral Neuroscience, Frontiers in Psychiatry and Frontiers in Cellular Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-065-0
DOI 10.3389/978-2-88966-065-0

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Citation: Pautassi, R. M., Pilatti, A., Bravo, A. J., Acquas, E., eds. (2020). Is Early Onset of Alcohol Use Associated with Later Alcohol Use?. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-065-0

Table of Contents

frontiers in Behavioral Neuroscience

EDITORIAL
published: 13 August 2020
doi: 10.3389/fnbeh.2020.00133

Editorial: Is Early Onset of Alcohol Use Associated With Later Alcohol Use?

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Keywords: alcohol, early onset, adolescents, adults, prenatal

Editorial on the Research Topic

Is Early Onset of Alcohol Use Associated With Later Alcohol Use?

Research in the neuroscience and epidemiology of alcohol use disorders (AUD) has significantly contributed to our understanding of why individuals use alcohol and why a significant fraction transitions to risky alcohol use or develop an AUD (Harris and Koob, 2017). Yet research efforts are still unraveling important scientific questions. One of those lingering, unanswered questions is the role that an early age of alcohol onset has on the probability of later engaging in risky drinking and/or developing an AUD. The promoting effect of an early alcohol onset on later alcohol use or AUD is an ubiquitous finding (Marshall, 2014). Whether or not the association implies a causal relationship is, however, still under investigation. It is possible that early alcohol exposure alters brain (Pascual et al., 2007) or social processes (Light et al., 2013) that, in turn, facilitate alcohol seeking and intake. Yet it is also possible that both events are explained by a common factor, such as a psychiatric precursor (Tedor et al., 2018). This Research Topic presents contributions toward bridging the gap between preclinical, clinical, and epidemiological research, highlighting new information to better understand the consequences of early alcohol exposure.

Binge or heavy episodic drinking (HED; $\geq$4 or 5 standard drinks in a short, usually $\leq$2 h, drinking occasion, for females and males, respectively) is a pattern of excessive alcohol intake prevalent in emerging adults and, particularly, in those with early drinking onset (Pilatti et al., 2017). The effects of binge drinking on the integrity of sub-cortical structures and on inhibitory control have been not extensively studied in humans. Moreover, there is dearth of longitudinal studies analyzing predictors of binge drinking in non-US or non-European samples. In this Research Topic Vera et al. identified, in 1240 Argentinian college students, six binge HED trajectories (Moderate Stable Frequency, Moderate Decreasing Frequency, Stable Infrequent, Decreasing Infrequent and No-HED), with membership in those trajectories with more frequent HED being promoted by a younger age of first drink or intoxication, greater perception of peer drinking frequency and higher levels of impulsivity. Two studies published on this Topic tested, via magnetic resonance imaging, binge-related alterations on the structural properties of the nucleus accumbens and caudate nucleus, and on the balance between response inhibition and alcohol-related processing. Suárez-Suárez et al. reported that, despite similar performance in an alcohol-cued Go/NoGo task, binge drinkers, but not their controls, showed increased frontal activity in the inferior frontal gyrus and in the anterior insula, probably reflecting a compensatory mechanism. The study by Sousa et al. revealed that the volume of the nucleus accumbens was increased in 20 college students (aged 18–23 years old) that reported having engaged

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *17 June 2020*
Accepted: *06 July 2020*
Published: *13 August 2020*

Citation:
Pautassi RM, Bravo AJ, Acquas E and Pilatti A (2020) Editorial: Is Early Onset of Alcohol Use Associated With Later Alcohol Use? Front. Behav. Neurosci. 14:133. doi: 10.3389/fnbeh.2020.00133

in binge drinking at least once per month (with a minimum duration of 10 months), when compared to their abstinent peers. These studies make significant contributions to our understanding of predictors and consequences of binge drinking/HED in young adults.

Adolescent vs. adult differences in response to binge alcohol are dissected in the contributions of Rosana Camarini's and Ricardo Pautassi's groups. The study by Carrara-Nascimento et al. addresses the impact of a binge-like dose of alcohol, administered 5 days after a sub-chronic alcohol exposure regimen, on adolescent or adult mice. Despite similar alcohol-induced locomotor sensitization at both ages, the study revealed age-related differences in prefrontal cortical and accumbal dopamine content, such that levels were lower in adolescent mice than in adult mice. Salguero et al., in turn, assessed the impact of binge-like voluntary drinking during adolescence or young adulthood on anxiety response, shelter seeking, and recognition memory at late adolescence, as well as on voluntary alcohol drinking. The study disclosed that rats binge-drink more in adolescence than in adulthood and that this pattern negatively impacts anxiety responses in late adulthood. Moreover, adolescent exposure to alcohol enhanced alcohol consumption in adulthood.

Exciting data pinpoint alcohol-induced neuroinflammation as a relevant factor in the pathogenesis of AUD (Kelley and Dantzer, 2011). The mini-review by Flores-Bastías et al. puts forward the hypothesis that adolescent alcohol exposure reduces the availability of the α-melanocyte-stimulating hormone, and hence the activation of hippocampal melanocortin four receptors. This leads to reduced activity of the brain-derived neurotrophic factor, which is associated with reduced neurogenesis and neuroinflammation. The authors suggest that this signaling pathway may link adolescent alcohol exposure to greater likelihood of AUD in adulthood. The authors conclude that the activation of melanocortin four receptors (for instance, via synthetic agonist peptides) should rescue these effects. Mira et al., on this Topic, further review the detrimental effects of developmental alcohol exposure, including alcohol-induced impairments in neuronal morphology and survival, on hippocampal function. The authors review critical data, with a focus in glutamatergic transmission, suggesting that prenatal/perinatal or adolescent alcohol exposure is more harmful to hippocampus-dependent cognitive abilities than adult alcohol exposure.

The other mini-review of this Topic, that of Towner and Varlinskaya, reviews the pre-clinical literature on the effects of adolescent alcohol exposure on later alcohol acceptance and associated behavioral alterations. It establishes that many, yet not all, of the studies reviewed showed increases in voluntary alcohol intake after adolescent alcohol exposure. The authors pinpoint that those studies that yielded high levels of alcohol intoxication at adolescence were more likely to report enhanced alcohol drinking in adulthood, than those that induced moderate or low levels of alcohol intoxication in adolescence. This suggests that pre-clinical models of adolescent alcohol exposure should focus on preparations that induce relatively high blood alcohol levels.

The first exposure to binge alcohol can also occur during gestation (Yates et al., 1998; Baer et al., 2003). This prenatal alcohol exposure (PAE) may disrupt normative brain development by altering the functioning of the immune system (Gauthier, 2015). The study by Doremus-Fitzwater et al. on this Topic indicates that PAE (i.e., an alcohol liquid diet given on gestational days 6–20; 35% daily calories from alcohol) may potentiate postnatal expression of Il-6 and IkBa after an alcohol challenge. These intriguing results suggest that PAE may exert long-term alterations in the neuroimmune gene expression of neuroinflammatory factors. PAE has also been associated with increased alcohol intake in the offspring, which could be the result of PAE enhancing stress sensitivity or anxiety. The latter was supported by Madarnas et al. in one of the articles of this Topic. Moreover, Madarnas et al. found that the anxiety-prone phenotype induced by PAE (forced access to 6% alcohol, for 20 days prior to mating and throughout pregnancy and lactation) was associated with alterations in the radial distribution of axons of the cingulate cortex and in the expression of serotonergic and cannabinoid receptors.

The postnatal increase in alcohol intake found after PAE can also relate to early associative learning comprising alcohol's sensory properties and pharmacological effects (Spear and Molina, 2005). The review by Gaztañaga et al. makes a critical appraisal of this possibility. The authors make the case that the neural underpinnings of this prenatal appetitive learning rely on fetal brain acetaldehyde activating the endogenous opioid system. The paper contributed by Miranda-Morales et al. provides a comprehensive and insightful review of the literature on this and other critical topics, related to prenatal exposure to moderate concentrations of alcohol (e.g., intubations of 1.0 or 2.0 g/kg/day, on gestational days 17–20). The review discusses the role played during the intrauterine life by olfactory and gustatory cues (toward the development of detection and discrimination capabilities) and that of such exposure in the recognition of the reinforcing properties of alcohol through associative memories.

While much research has examined the impact that early drinking has on alcohol dependence, few has examined whether this link depends on cross-national drinking context and policies. In their study, Conde et al. found a moderate/strong positive association between early drinking and dependence among participants across 169 countries; however, the strength of this association was context-specific, based on normative drinking practices and linked to country level income and gender. Specifically, strong relationships between early drinking and alcohol dependence were present among countries where abstention or low infrequent drinking seems to be normative, while no significant relationships were found in countries where drinking is the common practice.

The findings and discussions presented in this Topic suggest that delaying age of first alcohol use is a deterrent of subsequent engagement in risky drinking and can protect from brain

neurotoxicity and insult. Moreover, the first exposure to alcohol can occur much earlier than in adolescence, i.e., during prenatal life, which is associated with multiple neurobehavioral deficits and alterations in postnatal reactivity toward the drug.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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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.

frontiers in Behavioral Neuroscience

REVIEW
published: 24 January 2020
doi: 10.3389/fnbeh.2019.00288

Effect of Alcohol on Hippocampal-Dependent Plasticity and Behavior: Role of Glutamatergic Synaptic Transmission

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *10 October 2019*
Accepted: *18 December 2019*
Published: *24 January 2020*

Citation:
Mira RG, Lira M, Tapia-Rojas C, Rebolledo DL, Quintanilla RA and Cerpa W (2020) Effect of Alcohol on Hippocampal-Dependent Plasticity and Behavior: Role of Glutamatergic Synaptic Transmission. Front. Behav. Neurosci. 13:288. doi: 10.3389/fnbeh.2019.00288

Problematic alcohol drinking and alcohol dependence are an increasing health problem worldwide. Alcohol abuse is responsible for approximately 5% of the total deaths in the world, but addictive consumption of it has a substantial impact on neurological and memory disabilities throughout the population. One of the better-studied brain areas involved in cognitive functions is the hippocampus, which is also an essential brain region targeted by ethanol. Accumulated evidence in several rodent models has shown that ethanol treatment produces cognitive impairment in hippocampal-dependent tasks. These adverse effects may be related to the fact that ethanol impairs the cellular and synaptic plasticity mechanisms, including adverse changes in neuronal morphology, spine architecture, neuronal communication, and finally an increase in neuronal death. There is evidence that the damage that occurs in the different brain structures is varied according to the stage of development during which the subjects are exposed to ethanol, and even much earlier exposure to it would cause damage in the adult stage. Studies on the cellular and cognitive deficiencies produced by alcohol in the brain are needed in order to search for new strategies to reduce alcohol neuronal toxicity and to understand its consequences on memory and cognitive performance with emphasis on the crucial stages of development, including prenatal events to adulthood.

Keywords: alcohol dependence, hippocampus, plasticity, glutamatergic synaptic transmission, neuronal toxicity

INTRODUCTION

Alcohol is the most common social drug used worldwide, with an average annual consumption of 6.2 L of pure alcohol *per capita* or 13.5 g of pure alcohol per day (WHO, 2014). Alcohol consumption in the population is influenced by different aspects, including the volume of alcohol consumed, the drinking pattern, and the age and gender of the drinker (Sloan et al., 2011; WHO, 2014; Chaiyasong et al., 2018).

Alcohol impacts the health of consumers in many ways, but the central nervous system is especially affected by alcohol toxicity. In numbers, 4% of the total deaths attributable to alcohol are related to the occurrence of neuropsychiatric disorders such as epilepsy, unipolar depressive disorder, vascular dementia, and Alzheimer's disease (Shield et al., 2013), and more importantly, 24.6% of the total burden of disease attributable to alcohol is related to neuropsychiatric disorders (WHO, 2014). During pregnancy, alcohol consumption drives to the incidence of fetal alcohol syndrome (FAS), a medical condition wherein children born from alcohol-drinking mothers present learning and memory deficits as well as problems with daily life skills, communication, and socialization (Koob and Le Moal, 2005; Merrill and Carey, 2016). Excessive alcohol consumption among adults produces brain abnormalities, including a clinical syndrome known as alcohol-related dementia (ARD), which is the most common cause of dementia in people younger than 65 years old (Harvey et al., 2003). ARD is poorly diagnosed and difficult to recognize because of the lack of a typical pathophysiological profile in people who suffer from it, and it is different from the Wernicke–Korsakoff syndrome, wherein thiamine deficiency explains the brain abnormalities (Moriyama et al., 2006; Ridley et al., 2013).

Alcohol affects several brain areas such as the prefrontal cortex, the corpus callosum, the cerebellum, and the hippocampus. Substantial evidence suggests that one of the main targets of alcohol toxicity in the brain is the hippocampus; indeed the alcoholic population shows neuronal loss and a reduction in total hippocampal volume as shown by magnetic resonance imaging (Jernigan et al., 1991; Harper, 1998).

The hippocampus is a structure located under the cerebral cortex in the limbic system. It has a unique horseshoe-like shape and contains two regions, the cornu ammonis (CA) and the dentate gyrus (DG). The CA is further divided into four zones, namely, CA1, CA2, CA3, and CA4, all of them principally containing pyramidal cells. The connectivity of these zones is especially depicted in a trilaminar loop, wherein afferences *via* the axons of the entorhinal cortex project into the DG. The granule cells in the DG project mossy fibers onto the dendrites of the CA3 pyramidal neurons, and the axons from the CA3 connect to the CA1 neurons in a so-called Schaffer collateral pathway. From there, signals leave the hippocampus to return to the respective sensory cortices.

The hippocampus is one of the most-studied brain structures and is involved in complex processes such as learning and memory, including recognition memory and spatial processing/navigation (Bird and Burgess, 2008; Stella et al., 2012). Evidence shows that the dorsal (posterior in human) hippocampus develops this function, and damaging this portion strongly impairs the acquisition of learning and memory tasks (Moser et al., 1995; Pothuizen et al., 2004). About spatial processing in the human and rodent brain, the hippocampus works beside the thalamus and cortical areas in the creation of a global positioning system through specialized cells called place cells (Bird and Burgess, 2008). Additionally, the hippocampus is involved in emotional behavior (Toyoda et al., 2011). Particularly, the hippocampus participates in the regulation of emotions by responding to positive emotional pictures or stimuli, including memories of past good moments (Santangelo et al., 2018), *via* connections with the amygdala (Guzmán-Vélez et al., 2016). These emotional aspects of hippocampal function are governed by the ventral hippocampus (Moser and Moser, 1998; Fanselow and Dong, 2010) which, working with the amygdala, mediates the response of the rodent in the fear conditioning paradigm (Anagnostaras et al., 2002).

All of these complex processes are related to changes in the strength of the response of the hippocampal circuits, which include interconnections of the CA3 pyramidal neurons with the CA1 region and the DG, representing an extensive region of excitatory glutamatergic synapses (Rebola et al., 2017). These changes in synaptic strength may involve the different forms of calcium-dependent synaptic plasticity known as long-term potentiation (LTP) and long-term depression (LTD), both of them being strongly related to the cognition processes (Stuchlik, 2014).

Studies in rodents have revealed different hippocampal alterations after alcohol administration (Tarelo-Acuña et al., 2000; Obernier et al., 2002; Morris et al., 2010; Zhao et al., 2013), generating a significant amount of evidence that supports the pathological features found in human brains. However, alcohol's effects on the hippocampal formation are dependent on the developmental stage, triggering different alterations during gestation, adolescence, and adulthood. In this work, we review published data, from early studies to current evidence, on alcohol's effects on the structure and the function of the hippocampus, including cognitive abilities, cell number, neuron architecture, and electrical properties and function, with special attention on the function closely related to the excitatory glutamatergic transmission. Considering gestational alcohol exposure through consumption or treatment in adolescent and adult rodents, cumulative evidence across decades increases our understanding of the fetal alcohol spectrum disorders (FASD) and the consequences of alcohol intake and abuse in the human population.

HIPPOCAMPAL EFFECTS OF ALCOHOL IN PRENATAL AND NEONATAL DEVELOPMENT INTO ADULTHOOD

In humans, alcohol consumption during pregnancy leads to FAS with effects on the memory and the learning abilities after birth, besides resulting in a very characteristic phenotype (Koob and Le Moal, 2005; WHO, 2014; Merrill and Carey, 2016). FAS is the most common form of cognitive disabilities not related to heredity, and it is part of a wide spectrum of disorders (FASD) caused by alcohol consumption during pregnancy. Besides brain structure abnormalities, children with FAS or FASD show impaired cognition and intellectual abilities and have deficient self-regulation and adaptive skills. Indeed these children are context-specific learners, i.e., they learn information in one context, and they cannot apply this knowledge in another context (Denny et al., 2017; Wilhoit et al., 2017).

Rodent models have been a widely used strategy to study the hippocampal defects produced by alcohol consumption during pregnancy. At the behavioral level, the alcohol treatment of pregnant rats during embryonic days 6–20 (E6–E20) increased the escape latency of pups in the Morris water maze (MWM), measured at postnatal day 22 (P22; Blanchard et al., 1987). MWM is a widely used cognitive task to assess hippocampal performance. The rodents search a hidden platform in a pool using visual cues in the room, and they must learn the location of the platform. Escape latency is the time it takes them to find the platform and escape from the water (Morris, 1984). Cognitive impairment by prenatal ethanol exposure in MWM is persistent in the older pups, P40, P60, and P90 (Gianoulakis, 1990), even when using modified MWM in both males (Matthews and Simson, 1998) and females (An and Zhang, 2013, 2015). However, in a model of ethanol exposure by vapor inhalation in a schedule of 6.5 h of exposition per day between E9 and E20, the adult offspring (P63) did not show a cognitive decline (Oshiro et al., 2014). Performance in the other cognitive tasks has shown alterations in avoidance learning (learning of behavior to avoid a stressful or unpleasant situation) and not in working memory (holding information for processing during short periods; Bond and DiGiusto, 1978). In the object–place paired-associate task, which evaluates spatial processing depending on the medial temporal lobe (hippocampal formation), gestational ethanol exposure impaired the performance of adult rats, which are unable to discriminate between two objects on the basis of their location in the field (Sanchez et al., 2019).

Brain development in rodents and humans differ in the highest velocity of brain growth. In rodents, the highest velocity occurs postnatally, in contrast to humans where this phenomenon occurs in the third trimester of development (Cudd, 2005). With this consideration, a single injection of alcohol in pregnant rats (E8) did not produce effects on spatial memory in the adult offspring, while a single alcohol injection during the postnatal period on P7 produced effects on spatial memory when measured at P98 (Sadrian et al., 2014), indicating a role of the developmental stage on effects of alcohol on hippocampal function (Lee et al., 2016; Breit et al., 2019). Vapor ethanol exposure in the perinatal period (P1–P8) impaired the performance of 5-month-old litters in a MWM probe trial, correlating with deficiencies in the gene expression of glutamatergic synapse proteins in the hippocampus (Zink et al., 2011). Accordingly, two intraperitoneal injections on E8 have indicated an impaired performance in the acquisition phase of MWM in the adolescent female mice, while the adolescent male mice did not show differences in performance. This procedure also showed differences in the shape of the brain regions, including the cerebellum and the hypothalamus, but not in the hippocampus, possibly explaining the mild effects of alcohol exposure on learning and memory at this developmental stage (Fish et al., 2016). Aside from that, the mild defect on MWM performance found in adolescent offspring was not observed in adulthood, indicating that alcohol exposure on E8 did not profoundly affect the hippocampal function (Fish et al., 2018). Further, the administration of two hypodermal injections of alcohol at P7 impaired the performance on the MWM in the training and probe trial of 11-week-old mice, which was a session without the platform (Lee et al., 2016). However, a disadvantage of this model in studying the effects of alcohol in brain development is that it is assumed that the placenta and the mother's alcohol metabolism do not have a relationship with the effects of alcohol in the fetus. It is also important to consider that, in pups injected at P7, the blood alcohol concentration was approximately 0.5 mg/dl, while in the dams which were also injected, the blood alcohol concentration was similar (0.5–0.6 mg/dl), and it is assumed that the fetus reached the same concentration (Sadrian et al., 2014). In a variant of contextual fear conditioning where the hippocampus and the medial prefrontal cortex are required in proper functioning, neonatal exposure to ethanol impaired the performance of the mice in the task, which could be explained by the alterations in the gene expression of the medial prefrontal cortex and not the hippocampus (Heroux et al., 2019), suggesting that the cognitive disabilities after ethanol exposure could not be entirely related to the hippocampal structure and function.

Chronic alcohol consumption during pregnancy permanently decreases the number of CA1 pyramidal neurons in the offspring of rats, with no changes in the DG granule cells (Barnes and Walker, 1981; González-Burgos et al., 2006), likewise with four intraperitoneal (i.p.) injections of ethanol on E7 (Diaz Perez et al., 1991). Furthermore, the DG had shown a reduction in cell number only after postnatal alcohol administration and at high blood alcohol concentrations (peak of 231 $\pm$ 32 mg/dl), suggesting that the CA1 pyramidal cells are more susceptible to alcohol damage than the granule cells in the DG (Miller, 1995). In contrast, the *ad libitum* consumption of alcohol did not reduce the CA1 cell number in adult rats after gestational alcohol intake (Lobaugh et al., 1991), probably because the rats do not reach greater blood alcohol concentration by *ad libitum* intake. When a binge drinking pattern of alcohol consumption is used on pregnant rats or newborn pups, there is a reduction in the cell density and number in the CA1, CA3, and DG regions only when alcohol is administered in the period equivalent to the last trimester in humans (P4 to P9 in rats) or all three trimesters (E1 to P9; Livy et al., 2003), and there are no significant effects on the cell number when alcohol is administered to the rats in the period equivalent to the first two trimesters (Maier and West, 2001), suggesting that the third trimester equivalent is the developmental period when the hippocampus is more susceptible to the effects of alcohol. Moreover, ethanol exposure during this developmental stage reduces the number of GABAergic interneurons in adulthood (P90), possibly contributing to cognitive impairment (Bird et al., 2018). Binge-like alcohol administration during the gestation period produces neurodegeneration through an apoptotic mechanism (Ikonomidou et al., 2000), which has also been observed by assessing neuronal death in rat primary hippocampal neurons obtained from fetuses exposed to alcohol during the gestation period (Akbar et al., 2006). Neuronal loss seems to depend on the blood alcohol concentration reached, even if the dose is small. That is, at a lower blood alcohol

concentration, the neuronal loss is also lower, and a high blood alcohol concentration produces a higher neuronal loss (Bonthius and West, 1990). On the other hand, the different areas of the hippocampus have shown different vulnerabilities to binge alcohol treatment, with the CA1 and CA4 regions being more vulnerable than the neurons in the CA3 or DG regions (West et al., 1986; Bonthius and West, 1990).

By analyzing neurogenesis in DG neurons after pre- and postnatal alcohol consumption, researchers have found interesting effects regarding 5-bromo-2′-deoxyuridine (BrdU, a marker of cell in active proliferation)-positive cells. The consumption of alcohol in pregnant female rats during the gestational period (E1–E21) did not produce a change in the number of BrdU-positive cells when litters were measured at 6 days old. However, there was a reduction in the number of cells co-labeling with BrdU and NeuN or BrdU and GFAP (Uban et al., 2010). When ethanol consumption was given in the third-trimester-equivalent in humans (P4–P9 in rats), there were less BrdU-positive cells in the 80-day-old rats. Upon calculating an estimate of new NeuN-positive cells per unit volume using BrdU-positive cells per unit volume, the volume density of double-labeled neurons was found to be decreased in the ethanol-treated animals analyzed at 50 and 80 days old (Klintsova et al., 2007). Using the 7-day-old CD-1 mice, a single subcutaneous injection of ethanol reduces the number of BrdU-positive cells in the 5-month-old mice in DG, when BrdU was injected 1 month earlier. The other effects found were reductions in DCX and PCNA immunoreactivity especially in the dorsal hippocampus, reduction in cells co-labeled with Sox2 and GFAP, and increased caspase-3 immunoreactivity in the subgranular zone of DG. All of these findings indicate that a single dose of perinatal ethanol diminishes the progenitor cells and thus reduces adult neurogenesis (Ieraci and Herrera, 2007). Pregnant mice administrated with alcohol for 10 days, starting from gestational day 7, produced a reduction in DCX-positive cells but not Ki-67-positive cells in offspring prenatally exposed to alcohol when analyzed at postnatal day 56, suggesting that prenatal alcohol exposure maintains the damage throughout the adolescent and adult lifespan (Olateju et al., 2018). This observation is also perceived in other species, as macaques prenatally exposed to alcohol showed decreased neurogenesis in the young adult stage (Fedorchak and Miller, 2019).

At the neuronal level, the structure is also altered by ethanol consumption during pregnancy. Prenatal alcohol exposure has shown a reduction in dendrite length in P14 mice (Davies and Smith, 1981), and ethanol exposure on P4–P9 increases the complexity of the apical dendrites in the CA1 pyramidal neurons measured at P9 (Goeke et al., 2018). The dendritic spines are the major structures of excitatory synapses, which receive inputs from the other neurons (Harris and Kater, 1994). The establishment of a mature dendritic spine allows a functional connection between the neurons, while alterations in the number, the density, or the proportion of mature/immature spines have been related to neurological disorders (Fiala et al., 2002). Alcohol exposure of pregnant rats also generates a reduction in the dendritic spine density in the hippocampal pyramidal neurons when pups were tested at P15 after four i.p. injections since the seventh day of gestation (Ferrer et al., 1988) or at P65 after weeks of alcohol treatment (Diaz Perez et al., 1991), and importantly, prenatal alcohol exposure also increases the immature spines and decreases the mature spine numbers at P30–40 (Tarelo-Acuña et al., 2000; González-Burgos et al., 2006). Nevertheless, in some models, at 2 months after birth (∼P60), the litters showed a recovery in the mature spines and the spine density (Ferrer et al., 1988; Tarelo-Acuña et al., 2000), which could be related with the differences in the time of exposure and the blood ethanol concentration reached. Two ethanol injections during E8 produce transient changes in the structure of the pyramidal neurons at P1, which are fully recovered at P10, including the dendritic spine density (Jakubowska-Dogru et al., 2017), suggesting the importance of the developmental stage in ethanol exposure and recovery. On the other hand, differences have been reported in the establishment of synapses, with a decrease in the total synapses, the simple synapses, and the symmetric synapses in the molecular layer of the DG at P30 (Hoff, 1988) as well as alterations in the mossy fiber topography at P60 (West et al., 1981). All of these evidences point to the fact that alcohol consumption during gestation alters synapse formation and maturation, and these alterations could remain during adolescence and adulthood.

Finally, the electrical properties and functions have been shown as altered under prenatal alcohol exposure. The litters exposed prenatally to alcohol and evaluated at P50 showed an impaired LTP induction and an epileptic behavior in the CA1 region of the hippocampus (Swartzwelder et al., 1988). The LTP impairment was also observed in the DG *in vivo*, although without changes in the input–output (*I*/*O*) curves (Sutherland et al., 1997; Patten et al., 2013), suggesting an impaired ability to evoke potentiation without changes in the basal synaptic transmission in the hippocampus. Neonatal ethanol exposure also impaired the LTP in neonatal rats only at a high ethanol concentration (300 mg/dl) and without changes in the basal synaptic transmission, suggesting defects in synaptic plasticity (Puglia and Valenzuela, 2010). Gestational alcohol administration impaired the LTP in P36 offspring, and depotentiation was facilitated in the male offspring, while enhanced LTP and suppressed depotentiation were seen in females, suggesting a gender-dependent differential effect (An and Zhang, 2013, 2015). In another independent study, LTP impairment on the DG was observed only in males, while LTP was enhanced in females in a protocol of alcohol intake throughout pregnancy and recorded during adolescence (P30; Titterness and Christie, 2012) or during adulthood (Sickmann et al., 2014) of the offspring. Contrary results in juvenile offspring are also available, showing LTP impairment on the DG in both sexes (Fontaine et al., 2019). Surprisingly, both studies used the same experimental procedure; however, the juvenile rats were assessed on different time points, P30–35 and P21–28, respectively, which could explain the opposite results on females. Further studies are necessary to address the sex differences on LTP after prenatal alcohol exposure. Alcohol exposure at three different times, equivalent to

the three human trimesters, and evaluated at adulthood show an impaired dentate LTP more dramatically than when it was administered during the second-trimester-equivalent, supporting the evidence of specific periods of susceptibility to the toxic effects of ethanol during development (Helfer et al., 2012).

In vivo recordings of CA1 neurons have identified changes in theta activity, a type of oscillatory pattern in the hippocampus when the rodent is in active motor behavior and exploration. After prenatal alcohol exposure, both an increase in theta activity during movement and a decrease in theta activity during stillness were observed (Cortese et al., 1997). The DG *in vivo* recordings have indicated that prenatal alcohol exposure decreases the excitability of granule cells, as indicated by the reduced correlation between field excitatory postsynaptic potential (fEPSP) and population spikes (PS) amplitude, and impairs the maintenance of LTP once evoked (Varaschin et al., 2014), confirming the observations obtained in *ex vivo* brain slices for CA1 and DG LTP.

In summary, alcohol consumption during pregnancy alters the development and function of the hippocampus. Hippocampal impairment persists during adolescence and adulthood in the alcohol-treated dam's litters. This hippocampal impairment could probably be due to a decrease in the number of neurons, especially in the CA1 (a cell population more vulnerable to alcohol), an altered dendritic structure, and a reduced number of synapses, which also correlate with the alterations in the electrical behavior of neurons unable to potentiate in response to the high-frequency stimulations.

ALCOHOL CONSUMPTION IN YOUNG RODENTS

Brain development persists during childhood and adolescence in mammals. Alcohol consumption is not only risky during the pre-natal stages, but adolescence is also a crucial period in the maturation of the brain and its circuitry, where alcohol toxicity could cause damage in a long-lasting way. Furthermore, adolescence is usually the age for the start of alcohol consumption and abuse in humans; so, it is important to know the consequences that this behavior can have.

Comparing adult and young male rats, alcohol treatment at 30 min before MWM testing impaired the rats' performance in the acquisition phase and during the probe trial of the MWM task only in the young animals (Markwiese et al., 1998), suggesting that adolescents are more susceptible to alcohol-induced hippocampal dysfunction than the adults. In another study, acute administration caused poor cognitive performance in MWM in both adolescent and adult rats. Nevertheless, hippocampal dysfunction lasts up to 25 days later in adolescent rats, in contrast to adults where re-testing did not show differences between saline- and ethanol-treated rats (Sircar and Sircar, 2005). In the same line of evidence, female young and adult rats showed an impaired cognitive performance in the acquisition phase of MWM, while only young rats displayed a poor cognitive performance during the probe trial (Sircar et al., 2009), supporting the hypothesis that the adolescent and young populations are more vulnerable to alcohol-induced hippocampal dysfunction.

The binge drinking pattern of consumption becomes important especially in the adolescent population (Kuntsche et al., 2005). This pattern of alcohol consumption leads to high blood alcohol concentrations (>80 mg/dl) in a very short time (NIAAA), as what occurs in typical teenage parties. The binge-like protocols trigger adverse effects on the central nervous system function and can produce significant consequences over time (Merrill and Carey, 2016; Tapia-Rojas et al., 2017). The binge-like alcohol treatment induced an impairment of spatial processing and recognition memory even at 1 week later from the binge episode, while cognitive performance is re-established at 10 weeks later from the binge episode (Silvestre de Ferron et al., 2016; Tapia-Rojas et al., 2018) or as soon as 14 days from ethanol withdrawal in a binge drinking model in female rats (Fernandes et al., 2018). The re-establishment of recognition memory performance has been recorded as soon as 3 weeks after the last alcohol exposure in a binge-like paradigm (Tapia-Rojas et al., 2018). Contrary results have been published upon using vapor chambers compared to binge alcohol exposure. Alcohol administration for 10 h per 3 days for 4 weeks revealed the absence of a cognitive impairment in the MWM acquisition or probe trials (Schulteis et al., 2008), as well as treatment for 16 h per day for 4 days (Van Skike et al., 2012). However, the working memory was effectively impaired using this methodology in the MWM (Schulteis et al., 2008), indicating no alterations in spatial processing that depend on the hippocampus, which is contrary to the observations under the other binge-like administration protocols cited before. As we previously mentioned on prenatal alcohol treatment, alcohol vapor inhalation did not induce a hippocampal impairment in cognitive tasks, raising the possibility that in some way opposite results can be related to the differences in the way of administration, the time needed to reach high alcohol concentrations in the blood, and the actual blood concentration reached.

In the same line, binge alcohol treatment in young rats decreased neurogenesis, and new cells died probably by necrosis, given the low percentage of TUNEL-positive cells compared with pyknotic nuclei, wich is a characteristic of dead cells (Morris et al., 2010). When the DCX marker was studied, a decreased DCX immunoreactivity was observed after 4 days of binge ethanol treatment and after 2 days of withdrawal, but not after 7 days of withdrawal. After 28 days of withdrawal, a reduction in BrdU-positive cells was observed, indicating a reduction in cell survival after ethanol exposure in the adolescent rats (Morris et al., 2010). Nevertheless, intraperitoneal injections of alcohol for 3 days increase the number of TUNEL-positive cells and the number of pyknotic cells (Jang et al., 2002), suggesting neuronal death through an apoptotic mechanism. These pieces of evidence suggest that adolescent alcohol treatment seems to induce neuronal death; however, whether the mechanism is apoptosis or necrosis has not yet been elucidated. McClain also showed the increase in BrdU immunoreactivity not only in the DG but in the hippocampus as well, where colocalization with Iba1 was strongly observed in the adolescent rats (McClain et al., 2011). In the other studies using adolescent rats, the number of

BrdU-positive cells was only higher than the control after 7 days of withdrawal in a 4-day binge protocol, and this result was confirmed with Ki67 immunoreactivity (McClain et al., 2014). Also, in this study, they found that the majority of newborn cells (NeuN-positive as well) were ectopically ubicated; therefore, the new neurons could not integrate properly in the hippocampal circuitry (McClain et al., 2014). Upon evaluating neurogenesis with another alcohol consumption paradigm, a liquid diet *ad libitum* for 2 weeks, there was a reduction in BrdU-positive cells in both males and females (Anderson et al., 2012). In rats, ethanol consumption every 48 h for 20 days (11 exposures at the end) produced a reduction in the DCX immunoreactivity 22 days after the last dose of ethanol in adolescent rats but not in adult rats. This pattern of alcohol intake produced no changes in the Ki67 immunoreactivity in adolescent rats and an increase in the cleaved caspase-3 immunoreactivity, suggesting a persistent loss of neurogenesis only in adolescents and not in adults under the same ethanol protocol (Broadwater et al., 2014).

At the structural level, binge-like treatment in rats produced an increase in the immature spine number with a concomitant reduction in the mature spine number in the CA1 pyramidal neurons (Risher et al., 2015), suggesting a reduction in the number of spines as well as decreasing maturation, which correlates with the poorest excitatory transmission in the hippocampus. Accordingly, the granule neurons in the dorsal hippocampus have shown a reduction in dendritic spine density in the adult rats submitted to intermittent ethanol exposure during adolescence. Concomitantly, a reduction in the mushroom dendritic spines, as well as in the long dendritic spines, was observed in these rats (Mulholland et al., 2018), indicating that adolescent ethanol exposure alters the structure of the dendritic terminals persistently to adulthood.

Concerning the electrical function of the neurons, in mature rats only high concentrations of alcohol (100 mM) produced a decrease in the population excitatory postsynaptic potentials (pEPSPs), while in the immature rats pEPSP changed in a dose-dependent manner, with a significant effect even at 10 mM alcohol (Swartzwelder et al., 1995b). Importantly, the effect of alcohol on pEPSPs that depends on the activity of the ionotropic glutamate receptor *N*-methyl-D-aspartate receptor (NMDAR), a major contributor to LTP and LTD, is not mediated by the subunit GluN2B, a subunit particularly sensitive to regulation (Swartzwelder et al., 1995a). Moreover, using adolescent brain slices (P21–P26), Mameli et al. (2005) showed that the currents through NMDAR are inhibited by alcohol in a dose-dependent manner (10–50 mM), but in neonate brain slices the NMDAR currents were inhibited only at a concentration of 75 mM. The subunit composition of the NMDAR changes during development, and probably this different composition of the NMDAR subunits could explain the way that the hippocampal circuit responds to alcohol at both stages. More recent studies, however, have shown that GluN2B in the extrasynaptic membranes regulates a greater number of proteins than GluN2B in the synaptic membranes in adult rats submitted to intermittent ethanol exposure during adolescence (Swartzwelder et al., 2016). In mice, the chronic intermittent ethanol exposure alters GluN2B interaction with proteins important for a mechanism of LTD dependent on the metabotropic glutamate receptors (Wills et al., 2017), raising a new possible mechanism for cognitive impairment provoked by adolescent alcohol consumption.

The alcohol-related effects are suggested to be dependent on how alcohol concentration is increased. The CA1 recordings of fEPSPs showed that 60 mM alcohol produced a blockage of LTP induction, but when alcohol was administered in a stepwise manner in 10-mM increments every 15 min until reaching 60 mM, there was no blockage of the LTP, possibly indicating tolerance. This tolerance seems to be related to the alterations in the intracellular calcium storages and/or metabotropic glutamate receptor function (Tokuda et al., 2007), adding a new step of complexity in the effects of alcohol on hippocampal function.

The *ex vivo* slices exposed to alcohol showed alterations in synaptic plasticity in the other regions of the hippocampus, such as the perforant path. In the DG, the application of 75 mM alcohol produced an inhibition of LTP and an inhibition of hyperexcitability after a paired pulse, inhibiting NMDAR signaling more than affecting the inhibitory gamma-amino butyric acid receptors (Morrisett and Swartzwelder, 1993), in contrast to presynaptic GABA-A receptors which are very sensible to ethanol in the hippocampus and regulate glutamate release (Wakita et al., 2012). When the CA3 neurons were analyzed as the postsynaptic compartment (stimulating the perforant path), 50 mM alcohol produced an important decrease in the amplitude of another ionotropic glutamate receptor, the α-amino-3-hydroxy-5-methyl-4-isoxasolpropionic-acid-mediated excitatory postsynaptic currents in neonatal slices, but not in young rats (Mameli et al., 2005), suggesting the differing effects of alcohol in the glutamatergic neurotransmission in the hippocampus during development. Paired pulse facilitation (PPF) showed a decrease in glutamate release in the CA3 region of the neonate rats, but not in the juvenile rats (Mameli et al., 2005), and in the CA1 region (Hendricson et al., 2004), which could probably be due to the action of alcohol on the N-type calcium channels (voltage-gated calcium channels; Mameli et al., 2005), altering the amount of glutamate released by an action potential. There are forms of LTP independent of the activity of the NMDAR, which are also inhibited by the alcohol and GABAergic transmission, which modulates this type of plasticity, mediating the inhibitory effect of alcohol at least in part (Izumi et al., 2005).

The animals treated with alcohol in a vapor chamber model showed that the neurotransmitter release is altered after 1 day of withdrawal, but not after 7 days of withdrawal as revealed by PPF. On the contrary, the synaptic strength is reduced after 7 days of withdrawal and not after 1 day of withdrawal as revealed in the *I*/*O* curves (Nelson et al., 2005), probably suggesting a time relationship between the presynaptic and the postsynaptic effects. Following a schedule of 2-days-on and 2-days-off intraperitoneal injections of alcohol for 2 weeks (binge-like pattern), alcohol provoked a decrease in the slope of the *I*/*O* curves at 1 week after the protocol ended and an impairment of PPF, indicating the pre- and postsynaptic effects of the treatment, which are compensated and re-established to control conditions

3 or 7 weeks after the protocol (Tapia-Rojas et al., 2018). The effect of only two episodes of alcohol intoxication has been studied, and two injections of alcohol at toxic concentrations abolished the LTD at 48 h after the protocol ended. A possible mechanism is a change in the composition of the NMDAR subunits, indicating a shift toward increased GluN2B subunit expression (Silvestre de Ferron et al., 2016; Drissi et al., 2019).

Alcohol administration in a binge-like protocol during adolescence changes the action of alcohol on the GABAergic transmission in the adult DG. Specifically, the adolescent alcohol treatment makes the extrasynaptic GABA-A receptors more vulnerable to alcohol in adulthood and makes the synaptic GABA-A receptors less sensitive to alcohol in adulthood (Fleming et al., 2012), indicating that adolescent alcohol exposure drives long-lasting changes in the GABA-A receptors and the DG. Importantly, this effect is specific to a binge-like alcohol treatment during adolescence, while the same results were not found in the rats treated during young adulthood or adulthood (Fleming et al., 2013). Additionally, this binge drinking pattern of consumption during adolescence produces long-lasting effects on the essential potassium currents in the inhibitory interneurons of the CA1 region (Li et al., 2013).

ALCOHOL CONSUMPTION IN ADULT RODENTS

The alcohol-related effects on hippocampal function have been widely studied in adult rodents under different paradigms and experimental designs which are reviewed here.

The administration of alcohol acutely before performing a cognitive test impairs the hippocampal function in adult rodents (2 months and beyond). The mice and rats showed an impaired cognitive performance in the radial arm maze (Gibson, 1985; Matthews et al., 1995; White et al., 1997), the T- maze (Givens, 1995), and the MWM (Shimizu et al., 1998), all of which evaluate spatial memory and thus hippocampal function (Olton and Samuelson, 1976; Morris, 1984; Poucet and Benhamou, 1997; Dubreuil et al., 2003), suggesting the altered neurotransmission in the hippocampus. The fear conditioning test is a behavioral test that evaluates hippocampal function without evaluating spatial memory but assesses the processing of contextual information (Chang and Liang, 2017). It has been reported that the hippocampus is involved in contextual and not tone cue conditioning during this type of test (Kim and Jung, 2006). Acute alcohol administration decreases the freezing time in the contextual conditioning phase of the test, but not in the tone conditioning phase of the test (Melia et al., 1996), revealing an alcohol-triggered hippocampal dysfunction in a task different from spatial memory.

Chronic administration of alcohol did not impair the cognitive performance in MWM treated for 26 or 30 weeks (Blokland et al., 1993; Lukoyanov et al., 2000). However, when the short-term (4 weeks) and long-term (36 weeks) modes of administration were compared, the longer period induced an impaired cognitive performance (Franke et al., 1997), suggesting that periods of consumption longer than 30 weeks are necessary to produce detrimental effects on the hippocampal function. This conclusion does not consider the possible effect of aging on the hippocampal function, and maybe there is a relationship between both processes. In a C57BL/6J mouse model of 3 weeks of chronic free choice, alcohol intake produced an impairment in the conditioning phase and in the context test of the fear conditioning test, but there were no effects on the NOR task nor the Barnes maze task (Stragier et al., 2015), indicating a mild cognitive impairment and hippocampal dysfunction.

Chronic alcohol administration has also been associated with impaired performance in the other cognitive tasks such as the Hebb–Williams maze (working memory assessment; Bond and Digiusto, 1976; Fehr et al., 1976), the radial arm maze (spatial memory; Gál and Bárdos, 1994), the spontaneous alternation paradigm (spatial processing), the attentional set shifting (cognitive flexibility, dependent of frontal cortex; Vedder et al., 2015), the step-down passive avoidance task, the Greek cross maze, and the Shuttlebox task (Farr et al., 2005), all of them of which are related with avoidance learning, which involves the limbic system including the hippocampus (Gabriel, 1993). Chronic intermittent ethanol exposure (CIE) in vapor chambers increases the anxiety-like behaviors in the adult male rats, possibly related to the alterations in the synaptic activity of the ventral hippocampus and not of the dorsal hippocampus (Ewin et al., 2019).

The chronic alcohol consumption in rats induced a reduction in the number of hippocampal pyramidal neurons and granule cells after several months of intake and withdrawal (Walker et al., 1980; Cadete-Leite et al., 1988; Paula-Barbosa et al., 1993; Franke et al., 1997; Lukoyanov et al., 2000). A decreased number of granule cells is evident as soon as after 4 months of alcohol treatment (Lukoyanov et al., 2000) and persists after 5 months of treatment and 2 months of withdrawal (Walker et al., 1980). However, as mentioned previously, cognitive decline is observed when chronic consumption is sustained in time, at over 30 weeks of treatment. Thus, the reduction in the number of granule and pyramidal cells does not necessarily correlate with the cognitive decline, suggesting that compensatory mechanisms are relevant to maintain the cognitive performance. Interestingly, chronic alcohol consumption during 9 months, followed by 3 or 6 months of withdrawal, produces neuronal loss in the hippocampus, although there was a slight effect of aging itself (Lescaudron and Verna, 1985). In a protocol of intermittent ethanol exposure in a 2-days-on and 2-days-off paradigm in rats, with evaluation at 1, 25, or 165 days after the last dose of ethanol, the DCX immunoreactivity decreased in the dorsal and the ventral hippocampus 25 days after the last dose of ethanol (postnatal day 80), the Ki67 immunoreactivity was decreased, and the cleaved caspase-3 immunoreactivity was augmented in both the dorsal and the ventral hippocampus (Vetreno and Crews, 2015). In a vapor chamber model, the Ki67 immunoreactivity was decreased 15 days after the cessation of the protocol, while the DCX was decreased 15 and 56 days after the protocol ended. The ethanol vapor paradigm produced a persistent reduction in the proliferating NPC, which were becoming immature neurons, leading to a severe loss of neurogenesis (Ehlers et al., 2013). Importantly, the loss of neurogenesis also has been documented in post-mortem samples retrieved from an adult human well

characterized for alcohol abuse (Dhanabalan et al., 2018; Le Maître et al., 2018), suggesting that adult alcohol abuse has a broad effect among species and therefore more pharmacological testing has to be done in rodents.

Furthermore, 4 months of alcohol intake did not produce a significant reduction in the CA1 neurons, but after 4 months of withdrawal it was possible to observe a reduction in the CA1 pyramidal neurons, indicating that cell loss could be mediated more by the withdrawal period than alcohol consumption itself (Phillips and Cragg, 1983). In mice, chronic alcohol consumption has shown cell death in the DG by an apoptotic mechanism, increasing the cleaved caspase-3 immunoreactivity, and which is decreased using memantine (Wang et al., 2018), an NMDAR inhibitor, suggesting excitotoxic cell death. Binge alcohol drinking in adults has also been associated with the degeneration of neurons. Intermittent intraperitoneal injection of alcohol for 1 month caused a reduction in the number of pyramidal cells in the hippocampal CA3 region (Lundqvist et al., 1995). Also, Fluoro-Jade B staining, which recognizes neurons undergoing degeneration, increased in the granule cells of the DG and the entorhinal cortex, the main afference of the hippocampus, indicating neurotoxicity caused by the binge alcohol treatment (Cippitelli et al., 2010b), and it is prevented using a group II metabotropic glutamate receptor agonist along with a recovery in reversal learning in the MWM task (Cippitelli et al., 2010a). However, the mechanisms underlying the neuronal cell death are not conclusive. Previously, it has been proposed that binge alcohol intake produces neuronal loss by necrosis 2 days after administration (Obernier et al., 2002), while the other reports showed an increase in caspase-3 immunoreactivity, which co-labeled with NeuN (Qin and Crews, 2012), indicating apoptosis. The administration of repeated cycles of the binge-like alcohol treatment provoked an increase in Fluoro-Jade B staining in the hippocampus, which decreased to control values 2 weeks after the last dose of alcohol, indicating a decrease in cell death along with the withdrawal (Zhao et al., 2013). In female rats, binge drinking has been associated with a reduction in the number of cells in the DG despite the fact that neurogenesis is increased in this hippocampal region and there is a lack of cognitive impairment in the MWM (West et al., 2019), suggesting that neuronal loss did not correlate with cognitive decline, and neurogenesis is not sufficient to recover the cell number in the DG. Thus, there is an important difference between chronic and binge alcohol consumption. The first one seems to produce neuronal loss during the withdrawal period, while binge alcohol consumption induces neuronal death because of alcohol consumption itself.

Several studies have documented changes at the synaptic level, like an increase in the dendritic length of the granule cells (Cadete-Leite et al., 1988; Durand et al., 1989; Paula-Barbosa et al., 1993), which returned to control values after a long withdrawal period (Cadete-Leite et al., 1989a; Paula-Barbosa et al., 1993), and an increase in the percentage of the plasmalemma of mossy fibers occupied by synaptic contacts (Cadete-Leite et al., 1989b; Paula-Barbosa et al., 1993). In agreement, an important finding was reported by Lukoyanov et al. (2000) who, despite observing fewer neurons in the hippocampus, found no change in the number of total synapses between the mossy fibers and the CA3 pyramidal neurons, suggesting that, after chronic alcohol consumption, there is a remodeling of synaptic connections that might compensate for the loss of neurons and that can be correlated with the absence of cognitive impairment as discussed earlier. In 1978, Riley and Walker, using a model of chronic alcohol exposure in mice (4 months of consumption and 2 months of withdrawal), observed 50–60% reduction in the dendritic spine number in the dentate granule cells and the CA1 pyramidal neurons in mice (Riley and Walker, 1978). Two years later, a conflicting evidence was reported using chronic alcohol consumption in rats treated for 4 months (Lee et al., 1981) or for 12 months with 6 months of withdrawal (Cadete-Leite et al., 1989a). However, more evidences have emerged, supporting the effects of chronic alcohol exposure on the dendritic spines. Chronic alcohol consumption or administration produced a reduction in the number of the dendritic spines in the posterior and the anterior hippocampus in mice (Lescaudron et al., 1989) and rats (McMullen et al., 1984) and in the CA1 region of the hippocampus (King et al., 1988). Interestingly, in all these cases, alcohol withdrawal led to a recovery in the hippocampus spine number compared to the control values (McMullen et al., 1984; King et al., 1988; Lescaudron et al., 1989). In contrast, in the granule cells of the DG, the treatment increased the dendritic spines, followed by a reduction in spines during the withdrawal period (King et al., 1988). A more recent report, using alcohol treatment in a vapor chamber with a CIE model, showed a reduction in dendritic complexity that persists after abstinence and increases in spine density, which is reduced after prolonged abstinence in the DG. In contrast, the CA1 and CA3 cells showed more dendritic arborization during CIE, followed by a reduction comparable to that in controls after abstinence. Spine density in the CA3 and the CA1 did not change after CIE, but there was a reduction in the density after abstinence. In spite of the lack of correlation between the architectural changes and the dendritic spine density, the NMDAR subunit composition changes more accurately with architecture, increasing after CIE and decreasing after withdrawal (Staples et al., 2015). The dendritic spine density decreases in rats after four cycles of binge alcohol treatment and three cycles of withdrawal. However, at 14 days after the last dose of alcohol, there was a recovery in the dendritic spine density in the hippocampus and the entorhinal cortex (Zhao et al., 2013). To date, there is limited information about the effects of binge drinking on the synaptic connections or the dendritic spines.

Electrical recordings in *ex vivo* brain slices from 150-g to 200-g rats (approximately 6 weeks old) have indicated that 100 mM alcohol in the recording solution decreases the magnitude of LTP in the CA3–CA1 hippocampal synapse, while 50 mM alcohol did not change the LTP evocation (Sinclair and Lo, 1986), indicating that the inhibition of potentiation is dose dependent. Also, the process is reversible because when alcohol is washed out, the LTP is recovered, achieving control values after a second stimulation (Blitzer et al., 1990). In the CA1 hippocampal field, 20 mM alcohol enhanced the inhibitory postsynaptic currents mediated by the GABA-A receptors, and

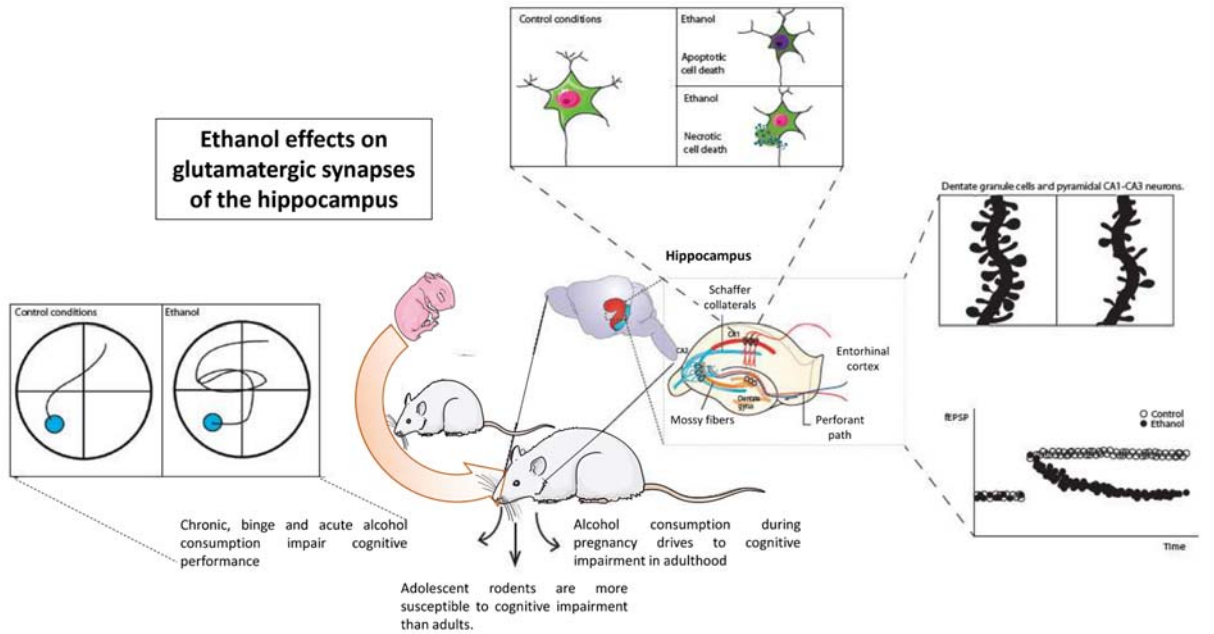

FIGURE 1 | Ethanol effects on hippocampal synaptic transmission and hippocampal function. Considering wide evidence, ethanol acutely alters the function of the hippocampus as measured in different cognitive tasks. A large amount of evidence shows alterations in structural plasticity inside hippocampal neurons, either dentate granule cells or pyramidal neurons. Glutamatergic transmission, especially that involving NMDAR function such as long-term potentiation, is also altered by ethanol.

this enhancement depends on the phosphorylation state of the receptor (Weiner et al., 1994).

The consumption of an aqueous solution of alcohol for 18 days prevented the induction of LTP in rat *ex vivo* slices (Johnsen-Soriano et al., 2007). The LTP impairment appears potentiated by the repeated withdrawals, not by a single withdrawal period, without changes in the *I*/*O* curves, in an oral model of alcohol consumption in rats (Stephens et al., 2005). Using mice, free-choice alcohol consumption for 21 days did not provoke LTP impairment in the LTP induced by 5 or 10 TBS bursts (Stragier et al., 2015).

Chronic alcohol consumption experiments using the CIE paradigm in a vapor chamber (12–14 days) have revealed a decrease in post-tetanic potentiation (PTP) and even the absence of LTP in the CA1 region. The PPF showed alterations only during PTP, indicating a reduced release of neurotransmitters. All of these findings were still present 1 day after withdrawal; however, at 5 days after withdrawal, at least the dendritic component of LTP showed recovery compared to the control group (Roberto et al., 2002). In agreement, the microdialysis experiments have shown that basal glutamate concentration is increased after 6 days of moderate doses of ethanol, indicating an increased neurotransmitter release under basal conditions (Chefer et al., 2011). This increase in extracellular glutamate in basal conditions could explain the alterations in the PPF. An increase in the basal glutamate release could reduce the number of exocytosis vesicles primed at the plasma membrane, explaining why a second pulse close enough is not sufficient to release more glutamate when the presynaptic calcium concentration is high.

Electrophysiological experiments performed *in vivo*, with the use of stereotaxic surgeries, have shown that alcohol i.p. administration acutely produced a decrease in the PS of the DG and CA1 neurons without effects on the fEPSPs. The firing rate was also decreased in both the DG and CA1 neurons, with no effects on interneurons (Steffensen and Henriksen, 1992), and LTP induction was altered in the DG (Steffensen et al., 1993). LTP is also inhibited *in vivo* by systemic alcohol administration at doses of 0.5 and 1.0 g/kg (Givens and McMahon, 1995). A 5% alcohol solution perfused intrahippocampally on the CA1 region produced a decrease in the neuronal firing and a slow-wave sleep pattern (Ludvig et al., 1995). Alcohol also decreased the NMDA-evoked activity in the hippocampal recordings *in vivo*; however, alcohol did not decrease the glutamate-evoked activity (Simson et al., 1993). A recording of hippocampal place cells during the radial arm maze procedure showed that a single alcohol injection (2 g/kg) 30 min before the recordings altered the place specificity of firing. The place specificity recovered after a withdrawal period of at least 24 h (Matthews et al., 1996).

CONCLUSIONS

Despite the discovery of the negative impact of alcohol on health, alcohol consumption persists at high rates worldwide. Hippocampal function in the temporal lobes has been described as an alcohol target, and rodent models have allowed understanding its effects.

Alcohol alters the cognitive abilities in rodent models. Alcohol administered acutely, chronically, or in a binge-like pattern severely impairs hippocampal function and cognitive performance in all stages of development. Prenatal alcohol exposure impairs the hippocampal functions, which lasts long into adolescence and adulthood, while adolescent alcohol exposure is more harmful to the cognitive abilities than adult alcohol exposure is, establishing that the developing brain is more vulnerable to the toxic effects of alcohol. Alcohol consumption triggers neuronal death in the hippocampal zones either by a necrotic or apoptotic mechanism, but conclusive results do not presently exist. The neurons alter their connections, including a decrease in the dendritic spine density and remodeling their synaptic contacts to maintain the number of connections between the neurons in response to chronic or binge alcohol patterns. Alcohol exerts its effects on the glutamatergic transmission by altering the NMDAR function and kinetics, while fewer variations in other fast glutamatergic currents have been reported. The structural changes observed are in concordance with the detrimental effects of alcohol on the LTP and the LTD, as well as other electrical measures of the glutamatergic transmission and the GABAergic transmission (**Figure 1**).

The evidence of the harmful effects of alcohol on the hippocampal formation is wide, and the structural and functional effects are understood. There is still considerable research needed to elucidate how short- or medium-term alcohol exposure can affect the long-term hippocampal performance and how they can contribute to the development of ARD. The complexity of alcohol research and its translation to human consumption falls on the number of variables to consider, such as age, pattern and time of ingesting, volume consumed, and physiological state of the subjects. For this reason, it is important to evaluate and introduce different models that can provide insight into how alcohol alters the cognitive processes in the brain and how it can perturb the cognitive state of consumers.

AUTHOR CONTRIBUTIONS

RM and WC: conception of the idea. RM: bibliographic research and main drafting. ML, CT-R, DR, and RQ: drafting and revising the article. WC: revising the article and correspondence.

FUNDING

This work has been supported by grants: Research Team Project in Science and Technology (ACT1411) to RM, CT-R, RQ, and WC; BMBF 20150065 (PCI) to WC; Fondo Nacional de Desarrollo Científico y Tecnologico (FONDECYT) 1140968 and 1170441 to RQ; Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) 1190620, Center for Excellence in Science and Technology (AFB 170005, PFB 12/2007), Sociedad Química y Minera de Chile (SQM) for special grant "The role of lithium in human health and disease," and FONDECYT 11181090 to DR.

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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.

frontiers in Cellular Neuroscience

MINI REVIEW
published: 28 January 2020
doi: 10.3389/fncel.2020.00005

Activation of Melanocortin-4 Receptor Inhibits Both Neuroinflammation Induced by Early Exposure to Ethanol and Subsequent Voluntary Alcohol Intake in Adulthood in Animal Models: Is BDNF the Key Mediator?

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OPEN ACCESS

Edited by:
Ricardo Marcos Pautassi, National Council for Scientific and Technical Research (CONICET), Argentina

Reviewed by:
Mercedes Lasaga, University of Buenos Aires, Argentina
Paula Abate, Medical Research Institute Mercedes and Martín Ferreyra (INIMEC), Argentina

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Received: *28 November 2019*
Accepted: *10 January 2020*
Published: *28 January 2020*

Citation:
Flores-Bastías O, Adriasola-Carrasco A and Karahanian E (2020) Activation of Melanocortin-4 Receptor Inhibits Both Neuroinflammation Induced by Early Exposure to Ethanol and Subsequent Voluntary Alcohol Intake in Adulthood in Animal Models: Is BDNF the Key Mediator? Front. Cell. Neurosci. 14:5. doi: 10.3389/fncel.2020.00005

The concept that neuroinflammation induced by excessive alcohol intake in adolescence triggers brain mechanisms that perpetuate consumption has strengthened in recent years. The melanocortin system, composed of the melanocortin 4 receptor (MC4R) and its ligand α-melanocyte-stimulating hormone (α-MSH), has been implicated both in modulation of alcohol consumption and in ethanol-induced neuroinflammation decrease. Chronic alcohol consumption in adolescent rats causes a decrease in an α-MSH release by the hypothalamus, while the administration of synthetic agonists of MC4R causes a decrease in neuroinflammation and a decrease in voluntary alcohol consumption. However, the mechanism that connects the activation of MC4R with the decrease of both neuroinflammation and voluntary alcohol consumption has not been elucidated. Brain-derived neurotrophic factor (BDNF) has been implicated in alcohol drinking motivation, dependence and withdrawal, and its levels are reduced in alcoholics. Deficiencies in BDNF levels increased ethanol self-administration in rats. Further, BDNF triggers important anti-inflammatory effects in the brain, and this could be one of the mechanisms by which BDNF reduces chronic alcohol intake. Interestingly, MC4R signaling induces BDNF expression through the activation of the cAMP-responsive element-binding protein (CREB). We hypothesize that ethanol exposure during adolescence decreases the expression of α-MSH and hence MC4R signaling in the hippocampus, leading to a lower BDNF activity that causes dramatic changes in the brain (e.g., neuroinflammation and decreased neurogenesis) that predispose to maintain alcohol abuse until adulthood. The activation of MC4R either by α-MSH or by synthetic agonist peptides can induce the expression of BDNF, which would trigger several processes that lead to lower alcohol consumption.

Keywords: melanocyte-stimulating hormone, MC4R, brain-derived neurotrophic factor, alcohol use disorder, alcoholism, α-MSH

Alcohol is the most commonly abused drug worldwide. The World Health Organization (WHO) placed alcohol abuse disorder (AUD) among the top risk factors for diseases leading to death worldwide (Lim et al., 2012; World Health Organization, 2014). As reported by WHO, the global effect of alcohol abuse is near 3.3 million deaths per year (approximately 5.9% of all deaths worldwide; World Health Organization, 2014). A study in the United Kingdom concluded that alcohol is the most noxious drug to society, above heroin and cocaine (Nutt et al., 2010). Alcohol abuse is associated with an increased risk of developing a variety of health issues including cancer and liver cirrhosis (Baan et al., 2007; Shield et al., 2013).

In addition to the various health problems associated with AUD, the excessive consumption of alcohol generates significant consequences in the brain such as neurodegeneration (Crews and Nixon, 2009), depression (Whiteford et al., 2013), anxiety (Kushner et al., 2000), memory impairment (Rose and Grant, 2010) and malfunction of the prefrontal cortex that leads to impaired executive function (e.g., planning, thinking, and judgment; Peterson et al., 1990). These alterations are especially important if excessive alcohol consumption occurs during brain development in adolescence, which would lead to long-term damage to brain structure and function (Crews et al., 2007). There is extensive evidence supporting the idea that the beginning of alcohol consumption in adolescence increases the risk of developing AUD in adulthood (DeWit et al., 2000; Dawson et al., 2008).

Although there are many neurobiological mechanisms involved in the development of AUD (i.e., motivation for alcohol consumption, establishment of chronic alcohol intake and relapse), the idea that neuroinflammation and oxidative stress play a role in the maintenance of chronic alcohol intake has recently gained relevance (Israel et al., 2017). It has been proposed that the activation of genes related to the innate immune system in the brain and neuroinflammation play a significant role in the establishment of addiction to alcohol (Coller and Hutchinson, 2012; Flores-Bastías and Karahanian, 2018). The activation of neuroinflammation would produce long-lasting neurobiological changes, as increased glutamate-induced hyperexcitability and excitotoxicity and decreased neurogenesis, which are related to addictive substances abuse; all these neurobiological changes are more evident if alcohol abuse begins at an early age (Crews et al., 2011). Although it was already known that alcohol consumption produced neuroinflammation (Blanco and Guerri, 2007), the first evidence that neuroinflammation would be directly related to alcohol addiction came in 2011 from experiments by Blednov et al. (2011), who demonstrated that the systemic administration of bacterial lipopolysaccharide (LPS) to mice increases voluntary ethanol intake for a prolonged period. Ethanol consumption disturb the intestinal barrier, allowing the passage of bacterial LPS into circulation (Ferrier et al., 2006). LPS inside the body induces the synthesis of tumor necrosis factor-α (TNF-α) by Kupffer cells, which is released from liver to the blood; TNF-α then crosses the blood-brain barrier and activates microglia and astrocytes TNF receptors to induce neuroinflammation (Qin et al., 2007; Crews and Vetreno, 2016). This state of neuroinflammation is maintained for several months (Qin et al., 2007), insinuating that there exist long-lasting mechanisms that perpetuate it. Accordingly, ethanol-induced neuroinflammation persists upregulated for a long period after alcohol withdrawal, whereas levels of peripheral inflammatory cytokines rapidly return to basal, indicating that neuroinflammation is maintained by a strong mechanism of self-perpetuation (Qin et al., 2008).

Besides the neuroinflammatory processes triggered by systemic LPS, the generation of reactive oxygen species (ROS) from ethanol metabolism in the brain may also contribute to neuroinflammation (Flores-Bastías and Karahanian, 2018). Microglia and astrocytes are key cells in the neuroinflammatory process, and they are highly reactive to ethanol exposure (Orellana et al., 2017), releasing proinflammatory cytokines and nitric oxide (Blanco and Guerri, 2007). Ethanol intake produces a potent activation of glial cells in both adult and adolescent rodents. Interestingly, withdrawal of alcohol intake by adolescent rodents does not completely reverse such activation when they reach adulthood (Evrard et al., 2006). The hippocampus is the brain region that suffers major pathological alterations due to chronic alcohol consumption (Franke et al., 1997). Astrocytes and microglia are highly activated in the hippocampus from alcoholics (He and Crews, 2008), increasing the release of pro-inflammatory cytokines which can lead to neuron death (Ward et al., 2009). In the case of adolescents, their hippocampus suffers greater damage than that of adults, since they are more sensitive to the EtOH-induced alterations of memory processes requiring hippocampal integrity (Markwiese et al., 1998; White and Swartzwelder, 2004).

Microglia, astrocytes, and neurons abundantly express NF-κB, an essential transcription factor that activates the genes of the innate immune response. NF-κB is also involved in ethanol-induced neuroinflammation (Zou and Crews, 2010). Ethanol increases cytochrome P4502E1 (Cyp2E1) activity (Lieber, 1999), and in turn, Cyp2E1 oxidation of ethanol increases ROS levels that activate NF-κB directly (Chandel et al., 2000), leading to the synthesis of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α (Cao et al., 2005). In addition, NF-κB activation induces NADPH oxidase expression (Cao et al., 2005), leading to the production of more ROS, which creates an "activation loop" that potentiates the innate immune response (Crews et al., 2011; Flores-Bastías and Karahanian, 2018). Accordingly, the daily administration of N-acetyl cysteine (a precursor in the formation of the antioxidant glutathione) to rats that have chronically consumed ethanol, has been shown to significantly reduce voluntary ethanol intake (Israel et al., 2017). Along this line, ibudilast, a drug that reduces TNF-α activity, inhibits by 50% chronic ethanol intake in high alcohol drinking rats (Bell et al., 2015). In addition to the intrinsic damage induced by ROS, leading to neuron death, NF-κB activation increase TNF-α, IL-1β, IL-6 and transforming growth factor β1 (TGF-β1) release, resulting in neuronal apoptosis (Crews et al., 2015). "The constant activation of neuroinflammation, together with the exacerbated production of ROS, would lead to neurodegeneration of key areas involved in excessive alcohol consumption. It has been proposed that, instead of simply

being a side effect of excessive alcohol consumption, neuronal damage associated with drinking may actually underlie some of the mechanisms that regulate the development of alcohol abuse disorder (Crews et al., 2015). Alcohol-induced cell death in regions such as the prefrontal cortex may lead to a lack of inhibition in subcortical reward areas such as the striatum, which in turn may reduce behavioral inhibition and increase motivation to drink. Repeated stimulation of the innate immune system during chronic or binge alcohol consumption may facilitate this process by decreasing inhibition of the mesolimbic reward system, thus increasing drinking (Crews et al., 2011, 2015; Flores-Bastías and Karahanian, 2018).

The hypothalamus is one of the brain regions that undergoes more pathophysiological changes during excessive alcohol intake (Barson and Leibowitz, 2016). In the hypothalamus, several orexigenic peptides (such as orexin, encephalin, and galanin) stimulate alcohol consumption (Rada et al., 2004; Schneider et al., 2007; Barson et al., 2010); on the other hand, anorexigenic hormones such as dynorphin, corticotropin-releasing factor and melanocortins inhibit alcohol drinking (Thorsell et al., 2005; Navarro et al., 2008; Barson et al., 2010). Melanocortins (MC) is a group of neuropeptides that are produced in the arcuate nucleus of the hypothalamus (Arc) and that include α-, β-, and γ- melanocyte-stimulating hormones (α-MSH, β-MSH and γ-MSH; Hadley and Haskell-Luevano, 1999). α-MSH is involved in the regulation of sexual behavior, food appetite and memory through its agonist activity on MC3R and melanocortin 4 receptor (MC4R) melanocortin receptors present in the hippocampus, paraventricular nucleus of the hypothalamus (PVN), ventral tegmental area (VTA) and nucleus accumbens (NAc; Mountjoy, 2010; Caruso et al., 2014). Interestingly, α-MSH has also been implicated in the modulation of ethanol intake (Olney et al., 2014). The administration of a nonspecific agonist of both MC3R and MC4R (Melanotan-II) reduces voluntary alcohol intake in mice (Navarro et al., 2003) and in alcohol-preferring rats (Ploj et al., 2002). However, when this agonist was administered to knock-out mice that lack MC4R no effect was observed, revealing that MC4R (and not MC3R) is responsible for reducing alcohol intake (Navarro et al., 2011). Accordingly, the administration of a selective MC4R-agonist synthetic peptide at the NAc and VTA lowered voluntary alcohol intake in rats (Lerma-Cabrera et al., 2012). Ethanol has direct effects on the levels of α-MSH in the hypothalamus, and these effects appear to be opposite depending on whether the exposure to ethanol is chronic or acute. Evidence indicates that chronic ethanol consumption reduces α-MSH levels in the hypothalamus (Rainero et al., 1990; Navarro et al., 2008; Lerma-Cabrera et al., 2013; Sprow et al., 2016), while an increase in α-MSH was observed in acute ethanol-treated rats (Shelkar et al., 2015).

The MC system has been linked for many years with anti-inflammatory effects in the brain (Macaluso et al., 1994; Orellana et al., 2017). MC4R is expressed in microglia and astrocytes (Caruso et al., 2007; Selkirk et al., 2007; Benjamins et al., 2013), suggesting that α-MSH may produce its anti-inflammatory effects *via* MC4R in these neuroinflammation-related cells (Caruso et al., 2004). The effect of MC4R activation on the reduction of neuroinflammation has been studied in models of brain injury other than ethanol intake, e.g., LPS-induced brain inflammation (Ichiyama et al., 1999a,b; Muceniece et al., 2005), cerebral ischemia damage (Giuliani et al., 2006; Spaccapelo et al., 2011) and spinal cord injury (van de Meent et al., 1997; Lankhorst et al., 1999). There is evidence that this ability resides in its capacity to decrease glial activation of NF-κB. Specifically, α-MSH and other synthetic melanocortin receptor ligands prevent IκBα phosphorylation and, consequently, inhibit NF-κB activation (Catania, 2008). In agreement, α-MSH decreases the release of pro-inflammatory cytokines such as TNF-α (Rajora et al., 1997; Wong et al., 1997; Delgado et al., 1998; Giuliani et al., 2006; Forslin Aronsson et al., 2007; Spaccapelo et al., 2011), enzymes as iNOS and COX-2 (Caruso et al., 2004) and inflammation mediators, like NO (Galimberti et al., 1999; Muceniece et al., 2006) in the brain. In addition to the inhibition of NF-κB activity, α-MSH also stimulates the production of the anti-inflammatory cytokines IL-10 from microglia and TGF-β from astrocytes, mediated by an increased expression of peroxisome proliferator-activated receptor-gamma (PPARγ; Carniglia et al., 2013). In recent work, we demonstrated that the activation of MC4R by a synthetic agonist peptide inhibits ethanol-induced neuroinflammation in rats (Flores-Bastías et al., 2019). However, the mechanism underlying this effect has not yet been elucidated.

Although we hypothesized that the activation of MC4R in the brain inhibits NF-κB activity and neuroinflammation induced by ethanol (Flores-Bastías and Karahanian, 2018; Flores-Bastías et al., 2019), MC4R elicits additional mechanisms that would help explain the effects of α-MSH or its synthetic analogs in reducing alcohol intake. After the pioneering work of Xu et al. (2003) who reported that the activation of MC4R induces brain-derived neurotrophic factor (BDNF) expression in the hypothalamus, it was determined that BDNF is a downstream effector of MC4R signaling in food intake control (Nicholson et al., 2007; Bariohay et al., 2009). Such signaling occurs through the cAMP-PKA-CREB pathway (Caruso et al., 2012); MC4R is a G protein-coupled receptor that activates adenylate cyclase leading to an increase in cyclic AMP (cAMP) levels, which activates protein kinase A (PKA). PKA then phosphorylates and activates the cAMP-responsive element-binding protein (CREB), which is a transcription factor. CREB mediates in several physiological processes in the central nervous system by the induction of different genes, including BDNF (Tao et al., 1998). Accordingly, CREB is activated by α-MSH-treatment in the hypothalamus (Sarkar et al., 2002; Sutton et al., 2005; Caruso et al., 2010).

BDNF is a neurotrophic factor that plays several roles in neurons growth and function. BDNF, through its receptor tropomyosin-related kinase B (TrkB), regulates CREB phosphorylation *via* the extracellular-signal-regulated kinases (Erk1/2) pathway (Bibel and Barde, 2000; Schinder and Poo, 2000), playing a key role in synaptic plasticity. BDNF signaling has also been implicated in alcohol drinking behavior, dependence and withdrawal (Davis, 2008), and its levels are altered in alcoholics (Heberlein et al., 2010). A BDNF gene polymorphism has been linked to a greater predisposition to develop AUD in humans (Uhl et al., 2001),

and a deficiency of the Bdnf gene causes increased alcohol intake in mice (Hensler et al., 2003). Accordingly, siRNA-mediated downregulation of endogenous BDNF in the striatum increased alcohol self-administration in rats, and subsequent infusion of exogenous BDNF reverted this response (Jeanblanc et al., 2009). It was suggested that BDNF would serve as a homeostatic factor in the hippocampus and striatum that somehow regulates ethanol intake (McGough et al., 2004). Changes in BDNF expression in the brain following chronic ethanol exposure play a role in the regulation of protracted alcohol consumption and withdrawal-induced anxiety (Pandey et al., 2006). Similar to that described above for α-MSH, exposure to ethanol has opposite effects on BDNF levels depending on whether the exposure is chronic or acute: after chronic alcohol consumption, BDNF gene expression in corticostriatal areas is downregulated (Logrip et al., 2009; Melendez et al., 2012). However, in the case of acute alcohol exposure, BDNF levels are increased in striatal and hippocampal brain regions (McGough et al., 2004; Jeanblanc et al., 2009; Logrip et al., 2009). Thus, it is conceivable that prolonged exposure to ethanol reduces α-MSH/MC4R signaling which would decrease BDNF expression, eventually leading to the establishment of a protracted alcohol consumption behavior.

What would be the mechanisms by which BDNF could regulate alcohol consumption? As mentioned above, neuroinflammation induced by ethanol is a process associated with the perpetuation of chronic alcohol intake, and the inhibition of such neuroinflammation lowers voluntary alcohol intake in animal models (Bell et al., 2015; Israel et al., 2017; Ezquer et al., 2018). Interestingly, BDNF triggers important anti-inflammatory and anti-apoptotic mechanisms in glial cells and neurons, respectively, and this could be one of the actions by which BDNF reduces chronic alcohol intake. It was reported that BDNF treatment decreases the levels of the proinflammatory cytokines TNF-α, IL-1β and IL-6 induced by bacterial components in the brain and increases the expression of the anti-inflammatory cytokine IL-10 (Xu et al., 2017). Similarly, BDNF suppressed TNF-α expression while increased IL-10 expression in the brain after ischemic injury (Jiang et al., 2011). Chronic alcohol consumption, in addition to producing neuroinflammation as we have already described, also leads to other important changes in the brain such as a reduction of neurogenesis in the hippocampus (Herrera et al., 2003; Crews et al., 2006; Zou and Crews, 2010). The hippocampus has been extensively examined because of its role in memory consolidation, along with the deterioration of cognitive function seen in alcoholics; interestingly, recovery from alcoholism is associated with increased neurogenesis in this brain region (Crews and Nixon, 2009). Because BDNF plays a fundamental

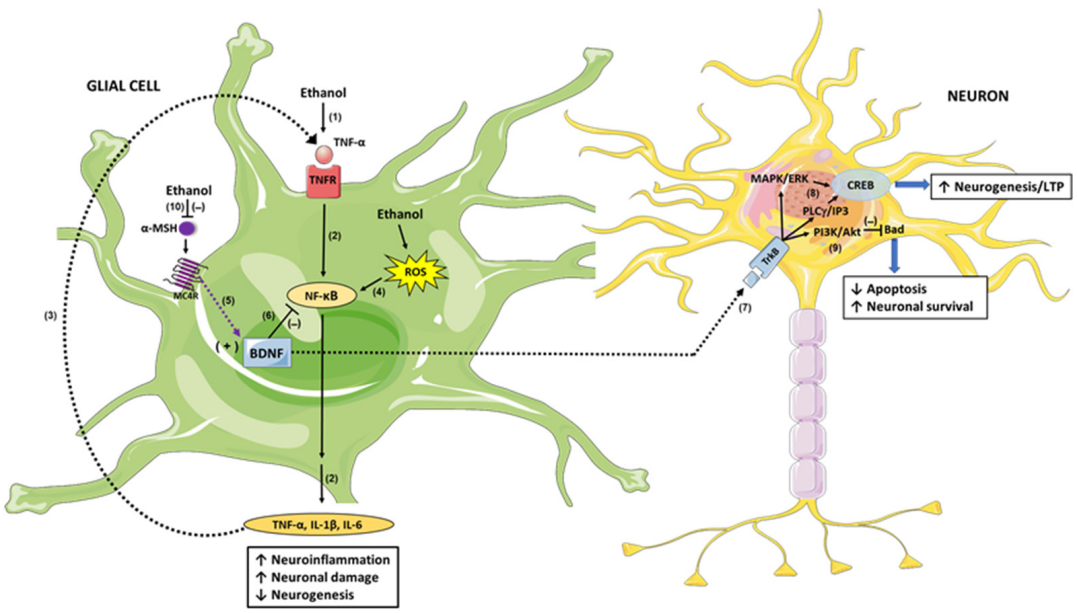

FIGURE 1 | Brain-derived neurotrophic factor (BDNF) mediation in the anti-inflammatory and neuroprotective response of α-melanocyte-stimulating hormone/melanocortin 4 receptor α-MSH/MC4R against damage produced by excessive alcohol consumption. Ethanol intake increases hepatic tumor necrosis factor-α (TNF-α) release into the blood (1); TNF-α is then transported across the blood-brain-barrier to glial cells and signals through Tumor necrosis factor receptor/Nuclear factor-κB (TNFR/NF-κB), inducing the expression of inflammation mediators such as IL-1β, IL-6 and TNF-α itself (2); these proinflammatory cytokines produce neuronal damage and reduce neurogenesis. The glial-secreted TNF-α binds to TNFR, creating an activation loop that potentiates the initial neuroinflammation response (3). Additionally, ethanol-generated reactive oxygen species (ROS) activates NF-κB directly, exacerbating the inflammatory damage (4). α-MSH/MC4R signaling induces BDNF expression (5), and BDNF inhibits NF-κB activity (6) thus inhibiting the neuroinflammatory response. In addition, BDNF secreted by glial cells acts in neurons through its TrkB receptor (7), signaling through MAPK/ERK and PLCγ/IP3 pathways (8) which in turn activate CREB, leading to increased neurogenesis and enhanced long-term potentiation (LTP). Additionally, BDNF/TrkB signaling activates PI3K/Akt pathway which inhibits the pro-apoptotic protein Bad, increasing neuronal survival (9). Ethanol decreases the activity of the central melanocortins system, reducing α-MSH levels and therefore MC4R activity (10); thereby, BDNF expression is downregulated averting its anti-inflammatory and neuroprotective activities.

role in neuroinflammation and neurogenesis, it is possible to speculate that activation of MC4R leads to the upregulation of BDNF in glia, which would decrease the neuroinflammation response (**Figure 1**). Also, BDNF secreted by glial cells would activate TrkB signaling in neurons, through the MAPK/ERK and PLCγ/IP3 pathways that upregulate CREB, leading to increased neurogenesis and enhanced long-term potentiation (LTP), which were reduced by chronic alcohol consumption (**Figure 1**). The latter would contribute significantly to improve cognitive disorders associated with alcohol consumption. Finally, BDNF signaling in neurons also activates the PI3/Akt pathway, which inhibits the pro-apoptotic protein Bad, thus decreasing ethanol-induced apoptosis and increasing neuronal survival (**Figure 1**). Additionally, the activation of MC4R in neurons would lead to an increased expression of BDNF through the PKA/CREB pathway in these cells. In this way, α-MSH/MC4R signaling increases BDNF secretion by neurons themselves, which would bind to their own TrkB receptors creating a self-activation loop that will potentiate the described neuroprotection mechanisms.

In summary, based on the background presented, we hypothesize that ethanol exposure during adolescence decreases the expression of α-MSH and hence MC4R signaling in the hippocampus, leading to a lower BDNF activity that causes dramatical changes in the brain (e.g., neuroinflammation, neuronal death and decreased neurogenesis) that predispose to maintain alcohol abuse until adulthood. The activation of MC4R either by α-MSH or by synthetic agonist peptides is able to induce the expression of BDNF, which would trigger several processes that lead to lower both neuroinflammation and alcohol consumption in adulthood.

AUTHOR CONTRIBUTIONS

OF-B and EK wrote the article. AA-C designed the figure.

FUNDING

This work was supported by DIUA149-2019 (Universidad Autónoma de Chile) and ACT1411 (PIA CONICYT).

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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.

frontiers in Psychiatry

ORIGINAL RESEARCH
published: 10 February 2020
doi: 10.3389/fpsyt.2019.01005

Increased Nucleus Accumbens Volume in College Binge Drinkers - Preliminary Evidence From Manually Segmented MRI Analysis

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OPEN ACCESS

Edited by:
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Reviewed by:
Giuseppe Carrà, University of Milano Bicocca, Italy
Adolfo M. García, Laboratory of Experimental Psychology and Neuroscience, Argentina

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Specialty section:
This article was submitted to Addictive Disorders, a section of the journal Frontiers in Psychiatry

Received: 12 October 2019
Accepted: 20 December 2019
Published: 10 February 2020

Citation:
Sousa SS, Sampaio A, López-Caneda E, Bec C, Gonçalves OF and Crego A (2020) Increased Nucleus Accumbens Volume in College Binge Drinkers - Preliminary Evidence From Manually Segmented MRI Analysis. Front. Psychiatry 10:1005. doi: 10.3389/fpsyt.2019.01005

Introduction: Binge drinking (BD) is characterized by high alcohol intake in a short time followed by periods of withdrawal. This pattern is very common during adolescence and early adulthood, a developmental stage marked by the maturation of the fronto-striatal networks. The basal ganglia, specifically the nucleus accumbens (NAcc) and the caudate nucleus (CN), are part of the fronto-striatal limbic circuit involved in reward processes underlying addictive behaviors. Abnormal NAcc and CN morphometry has been noted in alcoholics and other drug abusers, however the effects of BD on these subcortical regions have been poorly explored. Accordingly, the main goal of the present study was to address potential morphological alterations in the NAcc and CN in a sample of college binge drinkers (BDs).

Method: Manual segmentation of the NAcc and the CN was performed in Magnetic Resonance Imaging (MRI) of 20 college BDs and 16 age-matched alcohol abstainers (18–23 years-old).

Results: A two-way mixed ANOVA revealed no group differences in the volumetry of the CN, whereas increased NAcc volume was observed in the BD group when compared to their abstinent control peers.

Discussion: These findings are in line with previous automatically segmented MRI reports highlighting abnormalities in a key region involved in drug rewarding processes in BDs.

Keywords: alcohol, binge drinking, college students, nucleus accumbens, nucleus caudate, striatum, MRI manual segmentation

Abbreviations: AC–PC, Anterior commissure–posterior commissure; AUD, Alcohol Use Disorders; AUDIT, Alcohol Use Disorder Identification Test; AUDs, individuals with alcohol use disorders; BD, Binge drinking; BDs, Binge Drinkers; CN, Nucleus Caudate; MRI, Magnetic Resonance Imaging; NAcc, Nucleus Accumbens; ODI, Orientation dispersion index; ROI, Region-of-interest; ROIs, Regions-of-interest; TIV, Total intracranial volumes; VBM, Voxel-based Morphometry.

INTRODUCTION

Binge drinking (BD) is defined as repeated and brief episodes of high alcohol ingestion -a minimum of four drinks for women and five for men- in about 2 h interspersed with intervals of withdrawal. This phenomenon is commonly observed among adolescents and young adults mostly in the western countries (1–5). Remarkably, the average age of primary alcohol experience seems to be at 12 years old, and 13 for the first intoxication episode (6). This behavior is particularly worrying considering that early age drinking is associated with a greater risk of engaging in BD or suffers from an alcohol use disorder (AUD) in the near future (7–9). Likewise, early age drinkers seem to exhibit a higher susceptibility to the deleterious consequences of alcohol on the brain. Accordingly, several studies in both animals and humans have highlighted the major neurotoxic effects of acute intermittent alcohol consumption in the adolescent brain (10–13), especially in the late matured structures such as the prefrontal cortex (PFC) (14–17). In this sense, the youth tendency to engage in BD during adolescence has been associated with a protracted maturing course of the regions comprising this circuitry (18, 19). Apparently, the immaturity of this particular network has been proposed as underlying the reduced ability to regulate behavior, the involvement in a spectrum of hazardous situations/practices such as BD, and, ultimately, the increased susceptibility to the reinforcing properties of alcohol and other drugs (20–22).

Interestingly, frontal cortical regions and its connections to subcortical limbic areas have been described as more vulnerable to alcohol toxicity in adolescence (23–25). Specifically, the fronto-striatal limbic circuitry, involving the PFC, the anterior cingulate cortex (ACC), the amygdala, and the striatum, is well acknowledged in the field of addiction considering its key role in the development and maintenance of addictive behaviors (26, 27). Particularly, the ventral striatum -which includes the nucleus accumbens (NAcc)- has been associated with the reinforcing properties of acute alcohol consumption, whereas the CN, which is part of the dorsal striatum, seems to be more closely involved in the compulsive drug seeking behavior observed in addiction (25).

Morphometric alterations within the fronto-striatal reward pathway, namely, in the striatum nuclei, have been documented both in non-dependent and dependent substance users (e.g., 28–30). Specifically, increased NAcc volumes were observed in current alcohol and cannabis users (28, 31), while dependent alcohol or cocaine consumers, which were passing through a detoxifying process when assessed, displayed reduced NAcc volumes in relation to their age-matched healthy controls (29, 32, 33). Additionally, mixed results were observed in the CN. Whereas some studies failed to found morphometric alterations in the CN of alcoholics (29), others reported decreased volumes in this subcortical region (34).

However, despite the relevance of the striatal nuclei in addictive-like behaviors, relatively few studies have addressed the neuroanatomical substrates of these subcortical structures in young BD adults. To the best of our knowledge, to date only two studies have reported NAcc disruptions in college binge drinkers (BDs) in comparison with light drinkers. As such, using magnetic resonance imaging (MRI), Howell et al. (31) reported greater NAcc volumes in college BDs. A similar pattern of results, although gender-specific, was found by Kvamme et al. (35), namely, increased NAcc volume in BD females in comparison with non-BD females. Regarding the CN, the findings are less consistent. While some studies failed to find alterations in the BDs' CN morphometry (36), others reported decreased CN volumes in this population (30, 35).

MRI-based measurements and reproducibility are influenced by methodological factors, i.e., the image acquisition protocol, the pre/postprocessing pipeline, and the morphometric assessment, -automated *vs* manual segmentation- (37, 38). The most commonly used methods to estimate brain morphometry rely on completely automated segmentation algorithms (39) such as the voxel-based morphometry (VBM) method (40) or whole automated brain/region-of-interest (ROI) parcellation protocols with tools such as the Freesurfer or FSL (39, 41). On the other hand, manually segmented methods have long been referred as the gold standard for structural neuroanatomical measurements, generally used in small ROI segmentations/assessments, mainly for subcortical regions (e.g., 42). Clearly, both procedures have pros and cons. Specifically, the manual tracing technique is very time demanding, and requests a highly user intervention computational cost. Additionally, the ROI has to be identified and traced at each slice, limiting the application of this type of analysis to large amounts of data. Also, the precision of the segmentation might be compromised by the intra- and inter-rater variability (41, 43). Automated methods can in part counter some of these limitations by, for example, reducing the rater's subjectivity and increasing segmentation accuracy, but they largely depend on the parcellation/segmentation packages selection and preprocessing steps applied (e.g., 44). In this sense, manual tracing has some advantages in relation to the automated segmentation procedures such as more rigorous control of inter-subject variability of the brain's size and shape, superior accuracy in volume's estimation, and increased segmentation precision, especially when the ROIs' anatomical boundaries are more difficult to determine as in the subcortical regions (37, 44, 45). However, despite its potential for detecting structural alterations in addictive behaviors including alcoholism (46–48) to the best of our knowledge no study so far has manually segmented the striatal nuclei in BDs.

In this sense, we aimed to explore the structural properties of the NAcc and CN, in a group of young college BDs comparing with a group of AACs. We hypothesized an increased striatal volume in the BD group, primarily in the NAcc, when compared to the alcohol abstinent controls (AACs), in line with the previously VBM findings (31, 35).

METHOD

Participants

Initial recruitment of the potential participants to be enrolled in the present study was carried out throughout an online survey and it was based on the current alcohol and other substances

consumption, and age range (18–23 years old). This first selection was based on the BD definition established by the National Institute on Alcohol abuse and Alcoholism (4) -a minimum of five standard drinks (four for women) in a short period of time (2 h)- at least once per month, with a minimum duration of 10 months for the BD group. As for the control group, the criterion defined was not consuming alcohol either now or in the past.

Subsequently, a clinical interview and a behavioral assessment were conducted to assess whether the preselected participants were eligible to participate in the study. The assessment protocol included the Portuguese version of the Alcohol Use Disorder Identification Test (AUDIT) (49, 50), the Alcohol Use Questionnaire (AUQ) (51), the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA), the Individual Assessment Module (IAM), the Family History Assessment Module (FHAM) (52), the Portuguese version of the Symptom Checklist-90 revised questionnaire (SCL-90-R) (53, 54), and the Edinburg Handedness Inventory (55).

Participants with history of traumatic brain injury or neurological disorder, personal and/or family history of DSM-IV-R axis I disorder or alcoholism in first-degree relatives, scores > 90 for the global severity index (GSI) or at least 2 symptomatic dimensions for the SCL-90-R, scores ≥ 20 in the AUDIT or AUD based on the DSM-IV-R criteria, regular or occasional use of other drugs including prescribed psychoactive substances (except occasional cannabis use), uncorrected sensory deficits or left handedness were excluded from the study. Tobacco consumption was not defined as an exclusion criterion since a high correlation between alcohol and tobacco consumption in adolescents and university students has been consistently reported in the literature (56–60).

Finally, 36 college students were included in the study. Twenty individuals were assigned to the BD group and 16 alcohol-abstinent individuals composed the AAC group. **Table 1** displays the demographics and alcohol-related characteristics for both groups.

Previous to the MRI scanning, individuals were asked to not engage in BD episodes for the three preceding days, not consuming alcohol for at least 12 h before the MRI assessment, and avoid caffeinated beverages ingestion and smoking for at least 3 h in advance.

The research protocol was designed considering the ethical principles for medical research involving human subjects of the World Medical Association (WMA) present in the Declaration of Helsinki (61) and approved by the Portuguese Bioethics Committee of the University of Minho. Participants were informed about the research procedure, gave written informed consent and received a financial stipend.

TABLE 1 | Demographic and behavioral data for binge drinkers and alcohol abstinent controls.

	BD N = 20 Mean (SD)	AAC N = 16 Mean (SD)	t (34)
% Male	50%	37,5%	z = -0.740
% Female	50%	62,5%	
% Caucasian	100	100	
Age	20.45 (1.60)	21.00 (1.71)	.99
Age of BD onset	17.45 (1.08)	—	
AUDIT (total score)	11.20 ± 3.25	.62 ± 1.20	-13.43***
Number of times of BD per month	3.57 ± 1.87	0	-8.54***
Number of months with BD pattern	35.90 ± 14.03	0	-11.44***
Grams of alcohol consumed per week	151 ± 44.27	0	-14.78***
Speed of drinking (gr/h during BD episodes)	34.50 ± 8.26	0	-18.69***
Percentage of times getting drunk when drinking	43.25 ± 20.41	0	-9.48***
Tobacco Smokers	7	0	
Occasional users of Cannabis	2	0	

AUDIT, Alcohol Use Disorders Identification Test; BD, binge drinking; AAC, alcohol-abstinent control; SD, standard deviation.
All p-values reported are for 2-tailed independent samples t-tests, except for the variable gender for which a Mann-Whitney test was calculated.
****P < 0.001.*

Magnetic Resonance Image Acquisition

Before the MRI acquisition all the participants were screened for possible contraindications that could interact with the scanner magnetic field (e.g. earrings, hair hooks, keys, etc.) and affect their safety. Participants were then familiarized with the scanner and instructed on the procedure. At the time of the acquisition, supported by both the hospital technician and the investigator, the participants were comfortably placed in the scanner table with head and foot support, and noise-reducing headphones. The study instructions were again revised and the participant was given a call button to activate an alert in case they needed help and wanted to finish the session.

Sagittal high-resolution 3D T1 weighted anatomical images were acquired in a Siemens Magneton TrioTim 3T MRI scanner (Siemens Medical Solutions, Erlangen, Germany) equipped with a 32-channel receive-only head coil, and software Version Syngo MR D12 applying a magnetization prepared rapid acquisition gradient echo (MPRAGE) ascending interleaved sequence and parameters: repetition time (TR) = 2,700 ms, echo time (TE) = 2.33 ms, inversion time (TI) = 1,000 ms, delay time (TD) = 1,600 ms, flip angle (FA) = 7°, 192 slices with 0.8 mm thickness, slab thickness = 153.6 mm, slice gap = 0 mm, in-plane resolution = 1x1 mm^2, matrix size = 320x310 and 256 mm field of view (FoV). The total acquisition time was 6.49 min.

Image Preprocessing

After the acquisition, all MRI scans were visually controlled to discard for critical head motion or brain lesions. After this verification, the 3D-Slicer Version 4.10 image-editing tool (http://www.slicer.org) was used to perform image segmentation. Manual tracing of the ROIs was performed on the T1 MRI native space. Each ROI was computed as the sum of all the voxels included in the specific ROIs under assessment. Total intracranial volume (TIV) was calculated as the sum of gray matter, white matter and CSF volumes using the 3D-Slicer Version 4.10 EMSegmenter tool.

Region of Interest Definition

The region of interest (ROI) included the NAcc and CN. The manual segmentation protocol was the one proposed by Levitt et al. (62). Accordingly, a large polygon was draw on the axial view of the anterior commissure–posterior commissure (AC–PC) plane, used as a landmark to trace the NAcc. To delineate the NAcc, we drew two intersecting lines, the first one skimming the superior surfaces of the caudate and putamen, and the second drawn along the plane formed by the internal capsule–putamen border and intersecting the first (forming an "X"). A vertical line from the intersection of this "X" was drawn to the ventrolateral border of the putamen, marking Point A. To define the dorsal boundary of the NAcc, the midpoint between the medial edge of the caudate and lateral edge of the putamen at the level of AC–PC plane was marked (Point B). The NAcc was drawn anteriorly until it was no longer present at or below the level of the AC–PC plane. The CN were measured bilaterally, using three orthogonal planes, in all slices in which they appeared. **Figure 1** shows the 2D reconstruction of both structures — see Coutinho et al. (63) and Levitt et al. (62) for more details regarding the anatomical landmarks. Manual segmentation was performed with 3D-Slicer (http://www.slicer.org).

Data Analysis and Procedure

One rater segmented the NAcc and CN of the total sample (36 participants). Other researcher with experience in manual segmentation traced the NAcc and CN of 11 randomly assigned cases, corresponding to 30.5% of the sample. Both raters were blinded to the participant's diagnosis.

The NAcc and CN were manually segmented in 18 slices following the protocol referred above. The volumes of each ROI were computed as the sum of all the voxels included in the specific ROIs under assessment. The NAcc and CN volumes, plus TIV, total gray and white matter volumes were then extracted and exported to a SPSS database and statistical analyses were conducted for the NAcc and CN volumes (left and right hemispheres), plus TIV, total gray and white matter volumes using SPSS package Version 20.0.

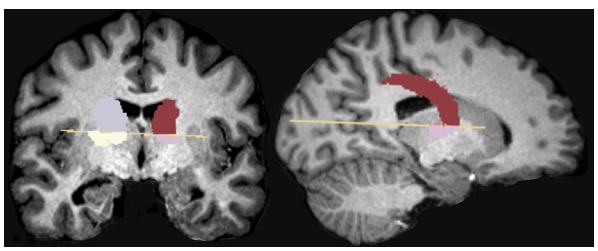

FIGURE 1 | Represents the NAcc and CN 2D reconstruction.

Statistical Analysis

The Intraclass correlation reliability between the two raters was assessed by Cronbach's alpha, and Student's t-tests were used to compare the TIV, total gray and total white matter volumes between groups. Statistical significance was defined as $p \leq 0.05$.

In order to correct for head size variation, the relative volumes were calculated for each ROI -absolute volumes divided by TIV and multiplied by 100.

A two-way mixed ANOVA was calculated for each ROI (NAcc and CN) with group (BD vs AAC) and gender (male vs female) as the between-subjects factors, hemisphere (left vs right) as the within-subjects factor, and age as covariate. When appropriate, degrees of freedom were corrected with the Greenhouse-Geisser procedure. False discovery rate (FDR) corrections were applied to the main and interactions effects, and post-hoc paired comparisons were performed with the Bonferroni adjustment for multiple comparisons (alpha level ≤ 0.05).

RESULTS

Control Measures

The Intraclass correlation reliability for the right and left CN and the right and left NAcc volumes by the two raters was good or excellent: .91 and .87, and .93 and .94, respectively.

Mean values for the TIV, total gray and total white matter volumes, absolute and relative volumes of the NAcc and CN for each group are presented in **Table 2**. Control analyses revealed no between group differences in the TIV [t (34) = 1.61, p = 0.12], total gray [t (34) = 1.38, p = 0.18] and white [t (34) = 1.02, p = 0.31] matter volumes.

NAcc Volume

A main effect of group revealed increased absolute NAcc volumes in the BDs [F (1,31) = 22.82, $p_{FDR} < 0.001$, $\eta_p^2 = 0.42$] when compared to their AAC peers (see **Figure 2**).

TABLE 2 | Means and SDs for the TIV, total GM and WM volumes, absolute and relative NAcc and CN volumes for each group.

				Volumes (ml) Mean (SD)							
GM	WM	TIV		NAcc Abs (ml)		NAcc Rel (ml)		CN Abs (ml)		CN Rel (ml)	
				Left	Right	Left	Right	Left	Right	Left	Right
BDs	897.23 (86.61)	507.57 (65.14)	1581.48 (158,15)	1.25 (0.15)	1.16 (0.19)	0.079 (0.008)	0.073 (0.012)	4.97 (0.58)	4.64 (0.64)	0.31 (0.02)	0.29 (0.03)
AACs	857.19 (85.94)	484.21 (71.58)	1496.19 (157.69)	0.93 (0.34)	0.80 (0.23)	0.063 (0.025)	0.054 (0.016)	4.65 (0.63)	4.33 (0.62)	0.31 (0.03)	0.29 (0.3)

AACs, alcohol abstinent controls; BDs, Binge Drinkers; CN Abs, Caudate Nucleus Absolute; CN Rel, Caudate Nucleus Relative; GM, Grey Matter; NAcc Abs, Nucleus Accumbens Absolute; NAcc Rel, Nucleus Accumbens Relative; TIV, Total Intracranial Volume; WM, White Matter.

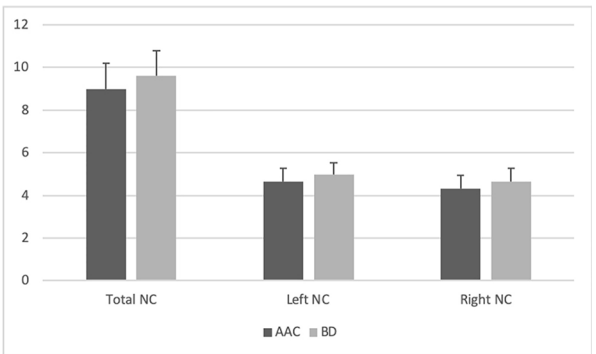

FIGURE 2 | Illustrates the CN mean volumes (ml) in the BD and AAC groups. Error bars represent standard deviations.

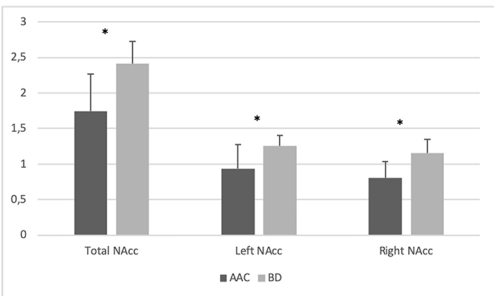

FIGURE 3 | Illustrates the NAcc mean volumes (ml) in the BD and AAC groups. Error bars represent standard deviations. *$P \leq 0.001$

Additionally, no significant Group x Gender [F (1,31) = 2.68, p_{FDR} = 0.30] or Group x Hemisphere [F (1,31) = 0.07, p_{FDR} = 0.93] interaction effects were observed. This pattern was also observed for relative NAcc volumes (to TIV), with a group effect [F (1,31) = 16.16, $p_{FDR} < 0.01$, $\eta_p^2 = 0.34$] and increased relative NAcc volumes in BDs, with no Group x Gender [F (1,31) = 1.01, p_{FDR} = 0.45] or Group x Hemisphere [F (1,31) = 0.01, p_{FDR} = 0.93] interaction effects being observed.

In order to control the potential effect of tobacco and occasional cannabis consumption in the results, the same analyses were conducted but excluding those BD participants who consumed tobacco and occasional cannabis. Similar significant group differences in the NAcc were found between BDs and AACs, both in the relative [F (1,23) = 9.43, p_{FDR} = 0.05, η_p^2 = 0.29] and absolute volumes [F (1,23) = 14.84, p_{FDR} = 0.01, η_p^2 = 0.39]. Additionally, no group differences were observed when comparing BDs without any other consumption and BDs with tobacco and occasionally cannabis consumption.

CN Volume

No significant between-group differences were found in the absolute or relative CN volumes [absolute: [F (1, 31) = 1.13, p_{FDR} = 0.74]; relative [F (1, 31) = 0.09, p_{FDR} = 1.00] (see **Figure 3**). Similarly, no Group x Gender [absolute: F (1,31) = 0.34, p_{FDR} = 0.84; relative [F (1,31) = 0.05, p_{FDR} = 1.00] or Group x Hemisphere [absolute: F (1,31) = 0.01, p_{FDR} = 0.97; relative [F (1,31) = 0.02, p_{FDR} = 1.00] interaction effects were noted.

DISCUSSION

This study assessed the NAcc and CN volumes in a group of college BDs in comparison with age-matched alcohol-abstainers using a manually segmented MRI protocol. Our results showed that BDs displayed greater bilateral gray matter (absolute and relative) volumes in the NAcc, whereas no between-group differences were observed in the CN, when compared to the AAC group.

The present findings are consistent with recent evidence showing increased NAcc volumes in college-aged BDs (31, 35). Specifically, Howell et al. (31) observed a greater NAcc volume in the BD group, relative to healthy controls, and suggested that this could represent a neuroanatomical immaturity associated with the binge alcohol use. Kvamme et al. (35) also observed an (gender-specific) increase in the NAcc volume of female BDs, a result that was again interpreted as a potential alcohol-induced deleterious effect on the neuromaturation trajectories of young BDs.

Developmental research has shown that subcortical gray matter volumes seem to peak during early adolescence, typically following an inverted U-shaped developmental trajectory (64). This natural decline in subcortical volume throughout adolescence – mostly driven by synaptic pruning (65) – has been reported as being disrupted by substance-induced toxicity, such as alcohol or cannabis (28, 31, 35). Likewise, maladaptive structural plasticity of NAcc neurons has also been proposed as a significant feature of excessive alcohol use, observed both in animal and human studies (66). In particular, exposure to intermittent alcohol administration in animal models has shown to increase dendritic branching and spine density in the mice NAcc (67, 68). Additionally, a recent study found higher orientation dispersion index (ODI) -a measure that captures the architecture of dendritic processes- in the ventral striatum of young adult BDs when compared to healthy volunteers (69). A positive association between the ODI and binge score (but not with the AUDIT) was further observed in BDs, suggesting that dendritic modifications –increased dendritic complexity – might be better explained by a binge pattern of alcohol consumption rather than by the severity of alcohol use (69).

Collectively, these studies open the discussion to two hypotheses. One suggests that BD at young ages may interfere with the neuromaturational processes (e.g., dendritic arborization, synaptic pruning) taking place in subcortical regions such as the NAcc. This interpretation of a neuromaturational delay has also been suggested from other studies showing increased gray matter volume in frontal regions in female (35, 70) or in both female and male BDs (71, 72). The other hypothesis suggests that pre-existing neuroanatomical differences may constitute a risk factor for BD, as documented by recent longitudinal studies (73, 74). Specifically, adolescents (females, but not males) with greater NAcc volume at baseline were more likely to binge drink 2 years later (73), thus suggesting that

delayed structural maturation of the NAcc may predispose towards excessive alcohol use in adolescence. Likewise, another study conducted by the same research group reported that adolescents with lower premorbid fractional anisotropy – an index of white-matter microstructure complexity – in pathways connecting the NAcc to frontal regions began BD sooner, suggesting again that a delayed maturation of accumbofrontal connections may represent a premorbid risk factor for earlier initiation of heavy alcohol use (74). Taken together, these mixed results come to confirm the need for new research, particularly longitudinal studies, in order to better disentangle the relationship between prefrontal/subcortical volume alterations and previous/subsequent alcohol use.

Regarding the CN, no significant between-group differences were observed in our study. Previous studies have provided little consistency when examining the potential abnormalities of this region in young binge or heavy drinkers. Specifically, in line with our findings, the co-twin study conducted by Wilson et al. (36) also failed to find changes in the CN morphometry of adolescent BDs, while others (30, 35) documented decreased CN volumes in this population. Accordingly, additional research is necessary to clarify the mixed CN findings reported in BD considering the involvement of this structure in the compulsive drug seeking behavior observed in addiction (75).

Overall, our findings add support to the assumption that morphological modifications in brain regions engaged in the addiction cycle (25) are present in BDs. Different roles of the dorsal and the ventral striatum have been proposed in the pathways that mediate the three stages of the addiction cycle. Specifically, the ventral striatum seems to be involved in the binge/intoxication phase characterized by the acute administration/use of drugs, which elicits a rewarding response (76), whereas the dorsal striatum appears to be recruited during the stimulus-response habit learning contributing to the development of compulsive drug-seeking behavior. Therefore, the instrumental function of each of these structures might be mediating the mixed results found in the studies as well as our observations. In addition, it has been reported that not only the NAcc, but also prefrontal regions of the fronto-striatal reward circuitry have shown to be structurally and functionally impaired in college BDs (e.g., 35, 73, 77–82), suggesting that alterations of the fronto-striatal reward network might be prompting or contributing to initiating/maintaining the binge pattern of alcohol consumption among youth.

Finally, this study displays some limitations that deserve consideration when interpreting the results. Firstly, the cross-sectional nature of the present study precludes us to determine whether differences in the NAcc volume precede alcohol use or are a consequence of BD. Secondly, the limited sample size of our study could also be an important factor that may undermine the reliability of the findings. Finally, the participants enrolled in our study are all college students, what might also influence the generalizability of our results.

In summary, the present study used a manually segmented MRI analysis to evaluate potential differences in the NAcc and the CN volumetry between college alcohol abstainers and age-matched BDs. Results revealed that the NAcc volume was significantly increased in the BD group in comparison with the AAC group. These findings seem to be in line with prior automatically segmented MRI studies carried out in young BDs and are suggestive of delayed structural maturation of the NAcc. Further research will be needed to determine whether these anomalies precede (and therefore constitute a risk factor for) alcohol use or instead are a consequence of BD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Portuguese Bioethics Committee of the University of Minho. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SS collaborated in the statistical analysis, interpreted the results, wrote the manuscript, carried out subject's recruitment and assessment, and participated in data acquisition and processing. AS coordinated data acquisition, collaborated in the data processing, statistical analysis and in manuscript writing. EL-C collaborated in interpretation of the results and manuscript writing. CB performed the manual segmentation protocol. ÓG collaborated in manuscript writing and AC designed the study, coordinated subject's recruitment, assessment and data acquisition, carried out the statistical analysis, and collaborated in manuscript writing. All authors read and approved the final manuscript.

FUNDING

This study was conducted at the Psychology Research Centre (PSI/01662), School of Psychology, University of Minho, and supported by the Portuguese Foundation for Science and Technology and the Portuguese Ministry of Science, Technology and Higher Education (UID/PSI/01662/2019), through the national funds (PIDDAC). This study was also supported by the project POCI-01-0145-FEDER-028672, funded by the Portuguese Foundation for Science and Technology (FCT) and the European Regional Development Fund (FEDER). SS was supported by the SFRH/BD/88628/2012, Doctoral Fellowship of the Portuguese Foundation for Science and Technology, co-financed by POPH/FSE through QREN. EL-C and AC were supported by the FCT and the Portuguese Ministry of Science, Technology and Higher Education, through the national funds, within the scope of the Transitory Disposition of the Decrete No. 57/2016, of 29th of August, amended by Law No. 57/2017 of 19 July.

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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.

frontiers in Behavioral Neuroscience

REVIEW
published: 05 March 2020
doi: 10.3389/fnbeh.2020.00026

Prenatal Alcohol Exposure as a Case of Involuntary Early Onset of Alcohol Use: Consequences and Proposed Mechanisms From Animal Studies

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Prenatal alcohol exposure has been found to be an important factor determining later consumption of this drug. In humans, despite the considerable diversity of variables that might influence alcohol consumption, longitudinal studies show that maternal alcohol intake during gestation is one of the best predictors of later alcohol use from adolescence to young adulthood. Experimental studies with animals also provide abundant evidence of the effects of prenatal alcohol exposure on later alcohol intake. In addition to increased consumption, other effects include enhanced palatability and attractiveness of alcohol flavor as well as sensitization to its sensory and reinforcing effects. Most of these outcomes have been obtained after exposing rats to binge-like administrations of moderate alcohol doses during the last gestational period when the fetus is already capable of detecting flavors in the amniotic fluid and learning associations with aversive or appetitive consequences. On this basis, it has been proposed that one of the mechanisms underlying the increased acceptance of alcohol after its prenatal exposure is the acquisition (by the fetus) of appetitive learning *via* an association between the sensory properties of alcohol and its reinforcing pharmacological effects. It also appears that this prenatal appetitive learning is mediated by the activation of the opioid system, with fetal brain acetaldehyde playing an important role, possibly as the main chemical responsible for its activation. Here, we review and analyze together the results of all animal studies testing these hypotheses through experimental manipulation of the behavioral and neurochemical elements of the assumed prenatal association. Understanding the mechanisms by which prenatal alcohol exposure favors the early initiation of alcohol consumption, along with its role in the causal pathway to alcohol disorders, may allow us to find strategies to mitigate the behavioral effects of this early experience with the drug. We propose that prenatal alcohol exposure is regarded as a case of involuntary early onset of alcohol use when designing prevention policies. This is particularly important, given the notion that the sooner alcohol intake begins, the greater the possibility of a continued history of alcohol consumption that may lead to the development of alcohol use disorders.

Keywords: prenatal, alcohol, learning, opioids, acetaldehyde, associative, reinforcer

OPEN ACCESS

Edited by:
Ricardo Marcos Pautassi, National University of Cordoba, Argentina

Reviewed by:
Marvin Rafael Diaz, Binghamton University, United States
Lindsay Halladay, Santa Clara University, United States

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *22 December 2019*
Accepted: *04 February 2020*
Published: *05 March 2020*

Citation:
Gaztañaga M, Angulo-Alcalde A and Chotro MG (2020) Prenatal Alcohol Exposure as a Case of Involuntary Early Onset of Alcohol Use: Consequences and Proposed Mechanisms From Animal Studies. Front. Behav. Neurosci. 14:26. doi: 10.3389/fnbeh.2020.00026

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) refers to the range of adverse effects that can occur in the children of women who consume alcohol during pregnancy (Riley and McGee, 2005; Manning and Eugene Hoyme, 2007). At the most severe end of the spectrum is Fetal Alcohol Syndrome (FAS), which may occur with prolonged consumption of relatively high amounts of alcohol (Jones and Smith, 1973; Jones et al., 1973). Prenatal exposure to alcohol may also produce either partial FAS (some of the diagnostic features occur) or may result in Alcohol-Related Birth Defects, or Alcohol-Related Neurodevelopmental Disorders (Stratton et al., 1996; Johnson et al., 2018) Interestingly, whilst the level of alcohol exposure may determine the severity of the observed effects, other cognitive, neuropsychological, and behavioral deficits have consistently been observed (Streissguth, 1986; Astley et al., 2009; Guerri et al., 2009; Kable et al., 2016; Temple et al., 2019).

Although not recognized as a symptom of FASD, there is evidence for an association between prenatal alcohol exposure and alcohol use disorders in offspring. Despite the considerable range of variables that might influence alcohol consumption by individuals, the few existing prospective longitudinal studies analyzing the relation between prenatal alcohol exposure and alcohol use by offspring all agree on one chief outcome: maternal alcohol intake during gestation is one of the best predictors of later alcohol use from adolescence to young-adulthood (Baer et al., 1998, 2003; Griesler and Kandel, 1998; Alati et al., 2006; Streissguth, 2007; Cornelius et al., 2016; Goldschmidt et al., 2019). Concurrently, numerous experimental studies with animals have shown increased alcohol intake in the offspring of dams that had consumed alcohol during gestation. These studies have been extensively reviewed in three previous publications, in which the effects of prenatal alcohol exposure on infantile, adolescent and adult alcohol acceptance and intake were described, analyzing the possible mechanism involved in those effects (Spear and Molina, 2005; Chotro et al., 2007; Abate et al., 2008). In the present review, we continue to explore this issue by analyzing specific research on the subject from the last 15 years, particularly those studies that have used animal models, focusing on the importance of mechanisms related to fetal learning about alcohol.

CLINICAL EVIDENCE

Most of the clinical evidence on the association between prenatal alcohol exposure and alcohol use disorders is derived mainly from a few longitudinal studies. The first of these is the "Seattle Prospective Longitudinal Study on Alcohol and Pregnancy," in which the effects of prenatal alcohol exposure were examined on a cohort of children born in 1974 to mothers selected as representative of the Seattle/King County population in the USA (Streissguth et al., 1981). In this study, among many other variables, alcohol use problems were measured in the offspring at three different ages: 14, 21 and 25 years. When subjects were 14 years old, three sets of data from 439 families were collected: adolescent alcohol use, family history of alcohol problems, and prenatal alcohol exposure history; and using these data they conducted correlational analyses. Prenatal alcohol exposure was found to be a better predictor of adolescent alcohol use than a family history of alcohol problems (Baer et al., 1998). When these same subjects were 21 years old, the families were re-evaluated, and it was reported that prenatal alcohol exposure was still strongly associated with a higher number of symptoms of alcohol dependence in early adulthood. This relationship persisted independently of the effects of a family history of alcohol problems, nicotine exposure, other prenatal exposures, and postnatal environmental factors including parental use of other drugs (Baer et al., 2003). At the age of 25, the association between prenatal alcohol exposure and adult alcohol use problems was still present (Streissguth, 2007). In all these cases the results were obtained after adjusting for maternal demographic characteristics, maternal use of tobacco and other drugs during pregnancy, and maternal and familial alcohol problems after birth. In line with those outcomes, alcohol exposure during gestation was also found to be a key factor for predicting alcohol use disorders in adults who were adopted at birth (Yates et al., 1998). In one study, 197 adoptees were evaluated for the use of alcohol, tobacco, and other drugs. Of those, 21 had received prenatal alcohol exposure and their outcomes were compared with those of 102 control adoptees who had received no alcohol exposure. The results showed that even when controlling for biological parental alcohol abuse or dependence, the prenatal alcohol exposure factor was still the best predictor of alcohol use disorders. This study highlights the relevance of alcohol exposure during prenatal development for alcohol abuse in adulthood, a relationship that appears to exist independently of confounding postnatal environmental variables (Yates et al., 1998).

Another follow-up study with participants from the "Mater–University of Queensland Study of Pregnancy and Its Outcomes" was designed specifically to analyze the association between maternal alcohol exposure and the onset of alcohol disorders. This study was conducted with 2,138 participants from a population-based birth cohort, born in Brisbane, Australia in 1981 (Alati et al., 2006). Mothers and their sons/daughters were followed from pregnancy to the offspring's early adulthood, and the onset of alcohol disorders was registered from adolescence to 21 years old. The results revealed that *in utero* exposure to three or more drinks containing alcohol was related to alcohol use disorders at the age of 21, increasing its risk by almost three times compared with subjects exposed to either smaller amounts of alcohol or those that had received no exposure. In addition, they reported that the sons and daughters of mothers who had consumed three or more glasses of alcohol during early pregnancy were almost four times more likely to show an early onset of alcohol disorders at the age of 21 than those whose mothers had consumed less than two drinks at any time. This association was robust, even after adjusting for a number of biological and environmental factors (Alati et al., 2006). In another publication, similar results were reported when subjects were 14 years old (Alati et al., 2008).

Another longitudinal prospective study aimed to analyze the relative contribution of familial risk and prenatal exposure to substance use in offspring. This study was carried out with a sample of 209 third-generation offspring of families from the area of Pittsburgh, USA, specifically selected for being at either high or low risk of developing alcohol dependence (O'Brien and Hill, 2014). High-risk families were selected based on the presence of two alcohol-dependent sisters and low-risk families were selected on the basis of having a minimal number of first and second-degree relatives with alcohol dependence. The results of this study showed that prenatal alcohol exposure increased the risk of alcohol use disorders in both high and low-risk participants, although high-risk mothers were more likely to use alcohol and cigarettes during each trimester of pregnancy. In addition, it was reported that among the high-risk offspring, the effects of prenatal exposure were more specific to the particular substance exposed, i.e., prenatal alcohol exposure was associated with alcohol problems in offspring, while cigarette exposure was associated with cigarette use.

Another study analyzed the link between maternal self-reported alcohol consumption during pregnancy and adolescent self-reported drinking in a sample of 185 mothers and their first-borns recruited from the New York State Cohort (Griesler and Kandel, 1998). Maternal drinking was assessed retrospectively, at an average of 3.3 years after delivery, with reports covering a period of 18 months (including pregnancy). Adolescent (age 9–17) life-time and current alcohol drinking data were obtained from self-reports. The results indicated that maternal alcohol drinking during pregnancy—particularly moderate to heavy consumption—was associated with the current drinking of their female offspring. No association was found, however, between maternal drinking, either during or after pregnancy, and alcohol drinking in sons.

Finally, a fifth longitudinal study was conducted with participants from cohorts of the "Maternal Health Practices and Child Development Project." These participants were recruited between 1983 and 1986, also from Pittsburg, USA, and they have been followed since the fourth gestational month. The data from this study demonstrated that the level of adolescent drinking (at the age of 16) was directly predicted by prenatal alcohol exposure, as well as lower levels of parental strictness and exposure to maltreatment and violence during childhood (Cornelius et al., 2016). These authors also reported that heavier drinking during adolescence is directly predicted by maternal alcohol consumption during pregnancy. A similar relation between these variables was observed when analyzing data collected during young adulthood, at the age of 22 (Goldschmidt et al., 2019). The results of these studies, together with the one described previously, provide clear evidence of the direct connection between maternal and adolescent drinking, which not only includes alcohol dependence and alcohol-related problems.

In sum, the outcomes of all these studies support the existence of an association between prenatal alcohol exposure and either early onset of alcohol drinking and/or with the development of alcohol use disorders in adolescence and young adulthood. In addition, most of these studies highlight a critical role for prenatal alcohol exposure in the idea of a causal pathway that leads to alcohol use disorders. Several mechanisms have been proposed to underlie the link between prenatal alcohol exposure and these consequences, although research studies at this level have primarily been conducted with laboratory animals.

EVIDENCE FROM STUDIES WITH ANIMALS

Experimental studies with animals provide abundant evidence confirming the results found in humans i.e., prenatal exposure to alcohol—in addition to producing numerous harmful effects—will, in most cases, be followed by an increased acceptance (i.e., attraction and consumption) of alcohol. These effects have been reviewed comprehensively in previous publications in which the outcomes of numerous studies with rodents exposed prenatally and perinatally to alcohol are described and analyzed, focusing on the behavioral effects of prenatal exposure, and in particular, on the factors that play a role in the postnatal response to alcohol (Spear and Molina, 2005; Chotro et al., 2007; Abate et al., 2008). **Table 1** includes the studies in rodents described in those reviews, as well as more recent publications, in which the effect of prenatal alcohol exposure on postnatal alcohol intake was assessed. As explained in those early reviews, increased alcohol intake has been observed after prenatal exposure to different doses of alcohol, ranging from relatively low to high alcohol concentrations. Those effects have been found in studies in which pregnant dams were given alcohol in a liquid diet made available for 24 h, as well as when alcohol was administered intragastrically in controlled amounts, modeling the so-called "binge drinking" of alcohol. In terms of the period of exposure, most of those studies show that exposure to alcohol during the entire gestation period of the rat (22 days), or only during the last 2 weeks, induces high alcohol consumption in the offspring. But even short binge-like exposures to relatively moderate alcohol doses, restricted to the final gestation days (GD 17–20), have systematically resulted in heightened alcohol intake. These results have been observed at different postnatal stages, infancy, adolescence, and adulthood. In general, there are more studies reporting this increased alcohol intake effect when tested early in ontogeny than in adulthood. However, there are some showing that this effect can be directly detected in late adolescence and even in adulthood, although in some cases postnatal re-exposure to alcohol seems necessary. The importance of other factors such as sex, genetic differences, and stress conditions at testing, on the detection of an effect of increased alcohol intake after prenatal alcohol exposure, has also been thoroughly discussed in those reviews (Spear and Molina, 2005; Chotro et al., 2007; Abate et al., 2008).

During the last decade, new studies showing evidence of an increased intake of alcohol following prenatal alcohol exposure in rodents have been added to those reviewed previously. Among all of this literature, those studies proposing and testing possible mechanisms by which fetal exposure to alcohol may increase the avidity for this drug are of particular interest for this review.

TABLE 1 | Studies in rodents measuring alcohol intake after prenatal alcohol exposure.

Reference	Prenatal period	Alcohol dose	Sex and test age (PD)	Outcome
Bond and Di Giusto (1976)	Whole gestation	Liquid diet with 6.5% (14 g/kg/day)	F56 and 70	Increased alcohol intake
Phillips and Stainbrook (1976)	Whole gestation plus lactation up to weaning	Chablis wine as sole liquid source	F170	Increased Chablis wine intake
Holloway and Tapp (1978)	GD 3 or 15 to PD 24, or to birth	Liquid diet 35% EDC	F-M28 and 70	Increased alcohol intake
Abel and York (1979)	From GD 10 to birth	1–2 g/kg i.g. daily	F150	No increase in alcohol intake
Buckalew (1979)	Whole gestation + lactation up to weaning	5% as sole liquid source	F28	Preference for alcohol over water
Randall et al. (1983)	GD 8–birth	Liquid diet 28% EDC (26–33 g/kg/day)	F-M25	Increased alcohol intake
Nelson et al. (1983)	Whole gestation	Not specified	F-M100	Increased alcohol intake, but under stress
Nash et al. (1984)	Whole gestation	10% as sole liquid source	M90	Increased alcohol intake
McGivern et al. (1984)	GD 7–birth	Liquid diet 35% EDC (14 g/kg/day)	F-M120	No increase in alcohol intake
Reyes et al. (1985)	Whole gestation	Liquid diet 20.9 EDC (16.85 g/kg/day)	F-M45	No increase in alcohol intake
Grace et al. (1986)	Either weeks 1, 2, 3, or whole gestation	2.8–3.5 g/kg/day	F-Maprox. 120	Increased alcohol intake
Hilakivi (1986)	Whole gestation	7% on weeks 1–2, and 12%, on week 3, as sole liquid source	M64	No increase in alcohol intake
Hilakivi et al. (1987)	Whole gestation	5% on week 1 and 10% on weeks 2–3	M90	Increased alcohol intake in ANA but not in AA rats
Molina et al. (1987)	GD 8	Two i.p. injections, 2.82 g/kg with 4 h–interval	F-M65–75	Increased alcohol intake
Lancaster and Spiegel (1989)	Whole gestation	Beer (50 ml/day or more, 9–11 g/kg/day)	F-M85	Increased beer intake
Molina et al. (1995)	GDs 17–20	1 or 2 g/kg i.g. daily	F-M15	Increased alcohol intake
Domínguez et al. (1998)	GDs 17–20	1 or 2 g/kg i.g. daily	F-M14	Increased alcohol intake
Honey and Galef (2003)	GD 7–birth	4% as sole liquid source	F-M26	Increased alcohol intake, but only if exposed on weaning
Chotro and Arias (2003)	GD 17–20	1 or 2 g/kg i.g. daily	F-M15 and 28	Increased alcohol intake
Arias and Chotro (2005a)	GD 17–20	2 g/kg i.g. daily	F-M14–15	Increased alcohol intake
Arias and Chotro (2005b)	GD 17–20	2 g/kg i.g. daily	F-M14–15	Increased alcohol intake
Pueta et al. (2005)	GD 17–20	2 g/kg i.g. daily	F-M15–16	Increased alcohol intake, but only if exposed on lactation
McMurray et al. (2008)	GD 5–20	Liquid diet 35% EDC + nicotine	F-M30–60	Increased alcohol intake, but only in female rats
Chotro et al. (2009)	GD 17–20	3 g/kg i.g. daily	F-M9–10 or 12–13	Increased alcohol intake
Youngentob and Glendinning (2009)	GD 11–20	Liquid diet 35% EDC	F-M30 and 90	Increased alcohol intake
Díaz-Cenzano and Chotro (2010)	GD 17–18 or GD 19–20	2 g/kg i.g. daily	F-M14 and 26–27	Increased alcohol intake in subjects exposed on GD 19–20
Shea et al. (2012)	Pre–pregnancy Whole gestation	5% + 1 g/l sucralose as sole liquid source	F-M40–45 to 70	Increased alcohol intake
Youngentob et al. (2012)	GD 6–10 or GD 11–20	6.7% as sole liquid source	F-M12–14	Increased alcohol intake
Abate et al. (2014)	GD 17–20	2 g/kg i.g. daily	F-M14–15	Increased alcohol intake, particularly in females
Díaz-Cenzano et al. (2014)	GD 19–20	2 g/kg i.g. daily	F-M14	Increased alcohol intake
Nizhnikov et al. (2014)	GD 17–20	1 g/kg i.g. daily	F-M14–15	Increased alcohol intake
Miranda-Morales et al. (2014)	GD 17–20	1 g/kg i.g. daily	F-M5	Increased alcohol intake
Fabio et al. (2015)	GD 17–20	2 g/kg i.g. daily	F-M37–62	Increased alcohol intake
Nizhnikov et al. (2016)	GD 17–20	1 g/kg i.g. daily	F-M14 (F1, F2 and F3)	Increased alcohol intake on all generations
Gaztañaga et al. (2017)	GD 17–20	2 g/kg i.g. daily	F-M14	Increased alcohol intake
Biggio et al. (2018)	GD 17–20	1 g/kg i.g. daily	M30–85 or 90–145	No increase in alcohol intake, but alcohol preference after maternal separation
Fernández et al. (2019)	Whole gestation + postnatal week 1	10% sole liquid source 22 h/day + water 2 h/day	M56–84	Increased alcohol intake from first testing trials, and potentiates isolation effects on alcohol intake
Gore-Langton and Spear (2019)	GD 17–20	2 g/kg i.g. daily	F-M35 and 56–60	Increased alcohol intake in adolescent and adult males
Wille-Bille et al. (2020)	GD 17–20	2 g/kg i.g. daily	F-M30–50	Increased alcohol intake and preference, but only males reared in enriched environment

GD, gestational day; PD, postnatal day; i.g., intragastric; F, females; M, males.

POSSIBLE MECHANISMS

On the basis of the results found in animal studies, a number of different mechanisms have been proposed to explain the increased consumption of alcohol that is observed following prenatal alcohol exposure. Some propose an indirect link between high alcohol consumption and other alterations induced by prenatal alcohol exposure, while others suggest more direct pathways between prenatal alcohol consumption and an increased acceptance of the drug.

Indirect Mechanisms

Genetic differences may lead to differences in susceptibility to the teratogenic effects of alcohol. For example, in a study using two lines of rats selected to differ in alcohol intake (AA, alcohol-preferring, and ANA, alcohol avoiding rats) prenatal alcohol exposure was found to differentially affect voluntary alcohol consumption (Hilakivi et al., 1987). This difference was explained by differences in alcohol metabolism between both ratlines. However, the authors did not offer any explanation for the connection between this differential susceptibility to the teratogenic effects of alcohol and the increased alcohol consumption. Epigenetic alterations have also been proposed as a mechanism by which prenatal alcohol exposure results in increased alcohol intake. The transgenerational transmission of the effects induced by prenatal alcohol exposure was investigated in a study including three generations of rats (Nizhnikov et al., 2016). Pregnant rats were administered with 1 g/kg of alcohol from GD 17–20, and the offspring of the first generation (F1) was tested in terms of alcohol intake and sensitivity to alcohol-induced sedation in comparison with water-exposed or untreated subjects. These F1 subjects were mated and their descendants (F2) were tested on those same measures and subsequently used to produce F3, which was also tested. The results of these tests revealed that alcohol intake increased in all three generations and that the effects on sedation were observed in F1 and F2, but not in F3. On the basis of previous research (Popoola et al., 2015), differences in maternal care were ruled out as a cause of this transgenerational transmission of the altered response to alcohol. However, in the discussion of the results, no alternative mechanisms were proposed to explain the interesting results observed in the F2 and F3 subjects. Transcript and epigenetic changes as a consequence of prenatal alcohol exposure have been described in several studies with animals (for a complete review, see Comasco et al., 2018), and these epigenetic modifications could underlie the results of the study conducted by Nizhnikov et al. (2016). Exploration of the mechanisms linking these genomic alterations and the increase in alcohol intake in subjects prenatally exposed to alcohol constitutes an interesting research topic that requires rigorous investigation. Recently, preliminary steps have been taken to clarify this issue with a study analyzing the protective effects of environmental enrichment upon modulation of gene expression, the anxiety response, and alcohol intake produced by prenatal alcohol exposure (Wille-Bille et al., 2020). The results showed that prenatal alcohol-induced upregulation in the kappa opioid receptor system mRNA levels in the amygdala, as well as prodynorphin mRNA levels in the ventral tegmental area, with the latter effect being linked to lower DNA methylation at the gene promoter. These effects were normalized by postnatal environmental enrichment manipulations. In addition, environmental enrichment also had a protective effect on alcohol intake, this effect being more marked in males than in females.

Prenatal alcohol has been found to alter the normal development of the neurochemical systems. Based on data showing that voluntary alcohol intake is partially regulated by the activity of the monoaminergic system (Ericson et al., 1998; Gonzales and Weiss, 1998) and that the acquisition of alcohol consumption habits is mediated by the dopaminergic mesencephalic system (Gianoulakis, 2001), it has been proposed that the altered alcohol intake after prenatal exposure is due to alterations in this system. Prenatal alcohol exposure has been observed to affect the development of the dopaminergic system (Shen et al., 1999; Aghaie et al., 2019), inducing, for example, hyperactivity (Cheng et al., 2018) together with sensitivity to the stimulant effects of alcohol (Becker et al., 1993; Barbier et al., 2009). These effects, together with other physiological and behavioral effects, have been linked to increased alcohol consumption in subjects prenatally exposed to alcohol.

Other neurochemical systems that have been shown to be related to motivational aspects of alcohol consumption, either directly or indirectly, and that are affected by alcohol exposure are GABA, serotonin, and the opioid system (Alfonso-Loeches and Guerri, 2011). Specifically, it has been demonstrated that the opioid system is involved in the increased alcohol intake observed in subjects exposed prenatally to a relatively low alcohol dose (1 g/kg; Nizhnikov et al., 2014). In this study, it was observed that prenatal alcohol exposure, in addition to increased alcohol intake in the offspring, induced changes in the kappa opioid receptor system. In particular, a decrease in synaptosomal kappa-opioid receptor expression was found in brain areas implicated in the response to alcohol. The authors suggest that these changes in kappa-opioid function and expression are involved in the enhanced postnatal alcohol intake observed following prenatal exposure.

Differences in the response to stress have been proposed as an alternative way to explain the increased consumption of alcohol following prenatal exposure. Some studies have linked stress and alcohol consumption *via* alterations in the development of the HPA-axis and the pituitary ß-endorphin system (Prasad and Prasad, 1995; Fahlke et al., 2000; Nash and Maickel, 2013). For instance, in one study, prenatal alcohol exposure was found to induce higher alcohol intake, but only when rats were tested after chronic stress (Nelson et al., 1983). Interestingly, in most studies, alcohol intake is tested in conditions of isolation, a stressful situation for rats that could facilitate the observation of differential effects of alcohol consumption. Prolonged isolation housing during early development has also been shown to induce augmented alcohol intake in rodents (Lopez et al., 2011; Kutcher et al., 2016). Additionally, in one study it was found that this heightened alcohol intake was facilitated in adolescent males exposed to alcohol during gestation and the first week of lactation (Fernández et al., 2019). In that case, males pre-

and postnatally exposed to alcohol showed significantly higher alcohol intake and increased alcohol preference in comparison with non-exposed controls during the first 5–6 testing sessions, but only if they had been housed in isolation conditions from weaning.

The anxiolytic effects of alcohol have also been considered as an explanation for the high alcohol consumption observed during stressful situations, particularly in adolescence (Spear and Molina, 2005). Recently, it has been found that adolescent rats exposed prenatally to alcohol were more sensitive to the social facilitation and anxiolytic effects of acute alcohol (Mooney and Varlinskaya, 2018). Another study examined the combined effects of prenatal alcohol exposure and postnatal maternal separation on HPA responsiveness, anxiety behavior, and alcohol intake, in male offspring (Biggio et al., 2018). The results of this study revealed that male subjects exposed prenatally to alcohol (1 g/kg) did not display an increased intake or preference for alcohol in comparison with non-exposed subjects or those that experienced only maternal separation. Maternal separation by itself was found to increase intake of low concentrations of alcohol, whereas adult males subjected to both treatments —prenatal alcohol and maternal separation—displayed an increase in anxiety-related behavior and an increased preference for alcohol at either low or high concentrations. The failure to observe an effect on alcohol intake in males following prenatal alcohol exposure is in accordance with the findings of another study in which a sex-dependent result was found after alcohol prenatal exposure, as a function of alcohol dose and age of testing (Chotro and Arias, 2003). This study found that whilst at infancy both males and females exposed prenatally to either 1 or 2 g/kg alcohol showed increased alcohol intake, at adolescence, only males exposed to the higher dose and females exposed to the 1 g/kg dose drank more alcohol than non-exposed subjects. It is possible that an increase in the effect of alcohol consumption might have been observed in the study by Biggio et al. (2018) if the female siblings had been included in the test.

Learning Mechanisms

Other mechanisms proposed to account for the heightened alcohol intake observed after prenatal exposure are based on the notion that the fetus may learn about different aspects of alcohol during exposure in the amniotic environment and this may have an impact on the postnatal response to the drug. Both human and animal fetuses detect chemosensory stimuli that enter the amniotic fluid from the maternal diet, and it has been shown that this experience may change the subsequent response to those flavors (Faas et al., 2000; Schaal et al., 2000; Mennella et al., 2001). Rat fetuses in the final days of gestation (GD 17 to birth) can acquire and express basic forms of non-associative and associative learning (Smotherman, 1982, 2002a,b; Stickrod et al., 1982; Smotherman and Robinson, 1985, 1988a,b; Gruest et al., 2004). Alcohol is one of those substances with chemosensory properties that is able to cross the placenta with ease and reaches not only the fetal tissues but also accumulates in the amniotic fluid, from where it is slowly eliminated (Chotro et al., 2009; Burd et al., 2012). Therefore, after maternal alcohol ingestion, the fetus is exposed to the flavor of alcohol as well as its pharmacological effects. Some studies explain postnatal increased alcohol intake in terms of a mere stimulus exposure effect, that is, familiarity with the alcohol flavor, or habituation to neophobia, which facilitates the initial acceptance of the particular chemosensory aspects of alcohol (Spear and Molina, 2005; Díaz-Cenzano and Chotro, 2010). However, this mechanism alone is not sufficient to explain the increased alcohol consumption observed when subjects are tested repeatedly and/or after a long period after the prenatal experience (Fabio et al., 2015; Gaztañaga et al., 2015).

Associative Learning Mechanisms

A mechanism that has been broadly investigated and has obtained abundant support in the last two decades, is one that links increased alcohol consumption to fetal associative learning about alcohol (Chotro and Arias, 2003; Arias and Chotro, 2005a,b, 2006; Chotro et al., 2007, 2009; Díaz-Cenzano and Chotro, 2010; Miranda-Morales et al., 2010; Youngentob et al., 2012; Díaz-Cenzano et al., 2014; Bordner and Deak, 2015; Gaztañaga et al., 2015). The working hypothesis of most of these studies begins with the assumption that the fetus acquires an appetitive conditioned response to alcohol by the formation of an association between the flavor of alcohol (the conditioned stimulus) and its pharmacological effects (the reinforcer).

The Role of the Opioid System

With regard to the pharmacological effects of alcohol, several studies have explored the implied role of the endogenous opioid system. This neurochemical system is known to play an important role in alcohol consumption behaviors (Gianoulakis, 2001, 2004) and in the mediation of the reinforcing effects of alcohol, particularly the mu-opioid receptor system (Acquas et al., 1993; Stromberg et al., 1998; Gianoulakis, 2001; Molina-Martínez and Juárez, 2020). Based on this body of evidence, the reinforcing properties of alcohol during gestation were tested by manipulating the prenatal opioid system. The results of several studies have shown that blocking the opioid receptor system with a non-selective antagonist (naloxone or naltrexone) during prenatal alcohol exposure prevented the observation of the increased alcohol intake effect in the offspring (for example, Chotro and Arias, 2003; Youngentob et al., 2012).

The fetal opioid system has also been found to be activated by the amniotic fluid, in the absence of alcohol. It has been proposed that the amniotic fluid contains a substance that stimulates the kappa-opioid receptors, known as KIF (kappa inducing factor), which is functional in the last two gestational days (GD 20–21; Méndez-Gallardo and Robinson, 2010). These researchers suggested that KIF could be the agent that mediates the preferences acquired by flavors experienced prenatally in the amniotic fluid, including the enhanced acceptance of alcohol observed after prenatal exposure to this drug. This hypothesis was tested in further studies in our laboratory. Taking into account that activity of KIF has been reported to start at around GD 20, the prenatal administration of alcohol prior to these days (GDs 17–18) would not be expected to produce the effect of increased intake, whereas this would be observed when alcohol exposure occurs on the following days (GDs 19–20). The results appear to support the hypothesis that KIF, and therefore, the

stimulation of kappa-opioid receptors, could play an important role as the reinforcer in this prenatal learning (Díaz-Cenzano and Chotro, 2010). In a second study, this hypothesis was tested from a more unambiguous perspective. Considering that KIF acts directly on kappa-opioid receptors, while the reinforcing effects of alcohol are mediated by the stimulation of mu-opioid receptors, the reinforcing effects of the amniotic fluid (with KIF) and alcohol were assessed by using specific antagonists for each receptor system. The results demonstrated that when prenatally blocking mu-opioid receptors during alcohol exposure, the usually observed effect of increased postnatal consumption of alcohol was completely abolished. However, the blockage of the kappa-opioid receptor system did not abolish this effect. These results allow us to rule out the possibility that the proposed effects of the amniotic fluid (and KIF) on the opioid system is the positive reinforcer responsible for the appetitive conditioned response. The pharmacological effects of alcohol on the mu-opioid receptor system were instead found to be critical for observing the increased consumption of alcohol after prenatal exposure (Díaz-Cenzano et al., 2014). Coincidently, an increase in the activity of mu-opioid receptors in the ventral tegmental area was reported after prenatal alcohol exposure, together with augmented alcohol intake in adolescence (Fabio et al., 2015).

Acetaldehyde as the Reinforcer

Once this was confirmed, further research aimed to clarify whether the reinforcer responsible for the activation of the opioid system is either alcohol itself or its first metabolite acetaldehyde. A growing body of research indicates that many of the deleterious effects of alcohol on gestation are actually produced by acetaldehyde (Sreenathan et al., 1982; Webster et al., 1983; Eriksson, 2001). As mentioned previously, during gestation alcohol freely crosses the placenta, reaching all fetal tissues to the same extent as maternal blood, including the brain (Zorzano and Herrera, 1989). From there, alcohol is eliminated, mostly unchanged, through maternal metabolism (Clarke et al., 1986). Fetal alcohol hepatic capacity is minimal or null, and therefore peripheral acetaldehyde is not produced by the fetus, while acetaldehyde produced by the maternal liver does not cross the placenta (Heller and Burd, 2014). However, in the fetal brain, acetaldehyde is produced in abundance from alcohol, mainly by the catalase system (Hamby-Mason et al., 1997). Several studies have demonstrated the important role of acetaldehyde in the pharmacological and behavioral effects of alcohol. It has also been found that acetaldehyde produced in the peripheral circulation (in the liver) and centrally (in the brain) have distinct and opposing behavioral effects: peripheral acetaldehyde induces aversive effects (Quertemont and Tambour, 2004) whereas central acetaldehyde is involved in the reinforcing appetitive properties of alcohol (for a review, see Hahn et al., 2006; Correa et al., 2012). Hence, the balance between peripheral and central acetaldehyde derived from alcohol consumption may be critical in determining the perceived effect of alcohol intoxication and may influence further intake of this drug.

Based on all of these facts related to alcohol metabolism, some studies have assessed the role of acetaldehyde on the increased alcohol consumption observed following its prenatal administration. In the infant and newborn rat, it has been found that acetaldehyde produced from alcohol by catalases in the brain is responsible for the reinforcing effects of alcohol (Nizhnikov et al., 2007; March et al., 2013a,b). The participation of centrally produced acetaldehyde has also been investigated in the prenatal period by administering to the pregnant rat the acetaldehyde sequestering agent D-Penicillamine together with alcohol. The results show that in the absence of acetaldehyde prenatal alcohol, exposure does not induce an increase in postnatal alcohol consumption. These results confirmed that acetaldehyde, and not alcohol, is the main reinforcer and that its production is critical for the occurrence of prenatal appetitive learning about alcohol (Gaztañaga et al., 2017; Chotro et al., 2019). Considering the outcomes of these studies, it could be hypothesized that the reinforcing properties of prenatal alcohol are produced by central acetaldehyde, which in turn stimulates the endogenous opioid system. This hypothesis is supported by studies showing that the reinforcing effects of acetaldehyde produced in the brain from alcohol may be mediated by the μ-opioid receptor system, and acetaldehyde has been found to stimulate the release of ß-endorphins (Font et al., 2013; Xie et al., 2013). In addition, the condensation product of acetaldehyde and dopamine, salsolinol (Ito et al., 2018), has been found in the fetal brain following chronic prenatal alcohol exposure (Mao et al., 2013). Salsolinol has been shown to be involved in the motivational effects of alcohol and its high intake and produces its effect by interacting with the μ-opioid receptors in the posterior ventral tegmental area (Xie et al., 2012; Quintanilla et al., 2014; Peana et al., 2017), Therefore, the monoamine system appears to be directly implicated in the reinforcing effects of alcohol, and, consequently in prenatal learning about alcohol; although the role of this system has not yet been fully investigated. This could yet prove to be the missing link between the reinforcing action of acetaldehyde and the activation of the opioid system.

The appetitive learning acquired *in utero* after alcohol exposure can also account for the increased preference or enhanced behavioral response to the odor of alcohol observed from newborns to adult rats (Youngentob et al., 2007; Eade et al., 2009, 2010; Middleton et al., 2009; March et al., 2013b; Gaztañaga et al., 2015). Interestingly, similar results have been reported in humans. For example, the newborns of mothers who frequently consumed alcohol during gestation responded to alcohol odor with more appetitive facial reactions than babies from control mothers who were infrequent consumers (Faas et al., 2000, 2015). In another study, it was found that young adults with prenatal history of alcohol exposure rated alcohol odor as more pleasant than non-exposed control subjects (Hannigan et al., 2015).

In addition to the enhanced response to alcohol odor, the prenatal experience has been observed to increase the reinforcing capacity of alcohol in operant conditioning tasks (March et al., 2009; Miranda-Morales et al., 2010; Gaztañaga et al., 2015); and has also been shown to interact with postnatal conditioning, potentiating appetitive learning about alcohol and retarding the acquisition of an aversion to this substance (Arias and Chotro, 2006; Chotro et al., 2009). The enhanced appetitive reinforcing properties of alcohol, together with the

development of tolerance and reduced sensitivity to alcohol's aversive effects, have also been proposed as mechanisms by which prenatal alcohol may lead to high postnatal alcohol consumption (Arias et al., 2008; Pautassi et al., 2012; Fabio et al., 2015; Gore-Langton and Spear, 2019).

These mechanisms are not mutually exclusive and the consistent outcomes of all the studies cited here suggest that they could be acting simultaneously to generate the augmented alcohol intake response systematically observed after exposure to alcohol during gestation. The increased alcohol intake described in many studies with human subjects exposed prenatally to alcohol may be partially explained by these same mechanisms that have been experimentally studied with rodents. For instance, those studies in which alcohol odor elicited positive reactions in subjects with prenatal alcohol exposure seem to support the idea of an appetitive response to alcohol acquired before birth (Faas et al., 2000, 2015; Hannigan et al., 2015).

CONCLUDING COMMENTS AND FUTURE DIRECTIONS

After reviewing all of the evidence from controlled experimental studies with animals, the connection between prenatal alcohol exposure and augmented alcohol intake during infancy, adolescence, and even adulthood appears to be clear. This link suggests that prenatal alcohol exposure increases the probability of early onset of alcohol use, which in turn has been described as a strong predictor of alcohol dependence (Grant, 1998). Although the causal relationship between adolescent-onset and adult alcohol use is still under debate (Prescott and Kendler, 1999), it is clear that adolescence is a vulnerable period for the neurobehavioral effects of alcohol (Spear, 2000, 2015). Unlike what occurs in adult subjects, initiation of alcohol use in adolescence has been found to accelerate the course of alcohol dependence, without the need for a long history of alcohol consumption (Clark et al., 1998; Spear, 2002). Therefore, any situation that favors the initiation of alcohol use during this period of development, such as stress or prenatal alcohol exposure, can be considered a risk factor for later alcohol use and misuse.

Thus, understanding the mechanisms by which prenatal alcohol exposure favors the early initiation of alcohol consumption may allow us to find strategies to mitigate the behavioral effects of this fetal experience. Moreover, it would be interesting to consider prenatal alcohol exposure as a case of involuntary early onset of alcohol use when designing prevention policies. This is particularly important if we assume that (as indicated by the longitudinal studies reviewed here), the sooner that alcohol intake begins, the greater the possibility of a long history of alcohol consumption and hence, the higher the likelihood of developing an alcohol use disorder.

Given these considerations, it is clear that more clinical and preclinical research is needed to explore prenatal alcohol exposure as a causal pathway leading to alcohol disorders in adolescence and adulthood. As discussed in this review, it has been well established that prenatal learning about the sensory and pharmacological properties of alcohol is a mechanism that plays an important role in facilitating the early onset of alcohol consumption. The opioid system has been found to mediate this prenatal learning, in which acetaldehyde acts as the main appetitive reinforcer. Future research should aim to find the link between acetaldehyde and the activation of the opioid system, with the dopaminergic system and/or salsolinol being the main candidates for this role. In addition to fetal alcohol learning, other identified coexistent and interacting mechanisms undoubtedly need to be investigated in more depth. For instance, stress is a factor that interacts with prenatal exposure to alcohol, facilitating in many cases the observed increase in alcohol intake in the exposed offspring. Further research is also needed to identify the causal processes by which prenatal alcohol-induced alterations in the activity of the HPA-axis drive the subject to consume more alcohol. Furthermore, it would be interesting to continue elucidating the role of the anxiolytic effects of alcohol on the increased intake response observed in subjects prenatally exposed to alcohol. Finally, the mechanistic connection between epigenetic modifications induced by prenatal alcohol exposure and the resulting changes in alcohol intake remains an underexplored but promising field of research, which needs to be addressed through rigorous research. In this regard, the first steps have already been taken, as shown, for example, in the results of a study already mentioned in this review (Wille-Bille et al., 2020).

AUTHOR CONTRIBUTIONS

MG, AA-A, and MC contributed equally to manuscript writing, revision, read and approved of the submitted version.

FUNDING

The research has been funded by the Basque Government (IT1341-19) to the research group (PI: Gabriel Rodriguez San Juan).

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 05 March 2020
doi: 10.3389/fnbeh.2020.00017

The Association Between Early Drinking and Dependence Varies by Drinking Context

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Evidence regarding the association between early drinking (ED) and later dependence is controversial. It has been alternately hypothesized that ED either plays a causal role in the development of dependence or that it is an early marker of increased psychosocial vulnerabilities. Despite a clear rationale for delaying youth consumption, it is important to discern this relationship. However, most epidemiological evidence comes from individual studies and high-income countries. If there is a causal link between ED and dependence, an association at the aggregate level would be expected. Furthermore, if the link is due to biological mechanisms, the association should be rather invariable regardless of the drinking context, while if the association is due to psychosocial factors, a wider variability is to be expected. We explored whether the association between ED and dependence varied across countries clustered by their shared contextual drinking characteristics. We used data from 169 countries from the Global Information System on Alcohol and Health of the World Health Organization: ED, alcohol dependence, heavy episodic drinking (HED), actual drinkers, and alcohol policy. To cluster countries by their shared drinking characteristics (prevalences of HED and actual drinkers, and alcohol policy), we used, sequentially, two multivariate data reduction techniques: a multiple correspondence analysis (MCA) and a hierarchic classification. To estimate the association between ED and alcohol dependence, beta regressions were performed, and then adjusted by country income-level and repeated by gender. The results indicated four country clusters: primarily abstainers (class 1), low drinking countries (class 2), high drinking countries (class 3), and very high drinking countries (class 4). Positive relationships between ED and alcohol dependence were found for all the countries in the world and for those in classes 1 and 2. No significant relationships were found for class 3 or class 4. These results were similar for males, but not for females, where no significant relationships were found after adjusting for income level. The association between ED and dependence varies according to the drinking context. Our findings either suggest that the ED–dependence association may be due to individual or environmental vulnerabilities that promote consumption outside cultural norms or that, if there is a causal link between ED and dependence, it is strongly moderated by psychosocial characteristics.

Keywords: alcohol, early drinking, dependence, alcohol policy, males, females

OPEN ACCESS

Edited by:
Adrian Jorge Bravo, College of William & Mary, United States

Reviewed by:
Jana Ruda-Kucerova, Masaryk University, Czechia
Mark Prince, Colorado State University, United States

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *13 October 2019*
Accepted: *23 January 2020*
Published: *05 March 2020*

Citation:
Conde K, Peltzer RI, Gimenez PV and Cremonte M (2020) The Association Between Early Drinking and Dependence Varies by Drinking Context. Front. Behav. Neurosci. 14:17. doi: 10.3389/fnbeh.2020.00017

INTRODUCTION

Evidence regarding the association of early drinking (ED) and the development of later problems is highly controversial (Frøydis et al., 2019). While many cross-sectional studies find an association between ED and later problems (e.g., dependence), longitudinal evidence accounting for confounders yields contradictory evidence (Connor et al., 2019). While there is a clear rationale for delaying consumption among youth, it is equally important to discern the nature of the relationship for theoretical and policy reasons. It has been alternately hypothesized that ED either plays a causal role in the development of later dependence or that it is simply an early marker of increased genetic and psychosocial vulnerabilities (Connor et al., 2019). Some authors have stated that, if there was a causal link between ED and dependence, an association at the aggregate level would be expected as self-selection and other biases present in individual-level studies would be absent. For this reason, some authors (Norström and Skog, 2001; Rossow, 2006) had pointed to the value of aggregate data for judging the plausibility of individual-level relationships when selection effects might be at play. However, as far as we know, available data from various countries, among them those from the ESPAD Study, failed to find such an association (Hibell et al., 2004; Kuntsche et al., 2016). Furthermore, if a causal relationship is to be assumed, and it is due to biological mechanisms (e.g., neurotoxic effects of ED), instead of social ones (e.g., through changing the social role of those drinking early; Frøydis et al., 2019), one would expect the association between ED and dependence to be rather invariable across countries; conversely, if the association is due to psychosocial factors, a wider variability among countries with different drinking contexts and policies is to be expected.

Most evidence about the association between ED initiation and the development of dependence comes from high-income countries, despite other world's regions depicting most of the alcohol-related harms. This gap in the evidence deepens the inequality between regions. Furthermore, the lack of studies from low- and middle-income countries has been indicated as a limitation of the available evidence, as the consequences of ED could vary cross-culturally (Maimaris and McCambridge, 2014; Frøydis et al., 2019).

Additionally, although alcohol use among females has increased over time and ED has grown among them, evidence regarding the link between ED and later trajectories of alcohol use and related problems between genders has also been contradictory (Tomek et al., 2016).

We aimed to explore whether the association between ED and dependence varies across countries clustered by their shared contextual drinking characteristics and national alcohol policies. If there is a causal link between ED and dependence, we expect to find an association at the aggregate level. Furthermore, if the causal mechanism is of a biological nature, we expect this association to be fairly even among clusters of countries unrestrictedly of their drinking context. Conversely, if the relationship is mainly due to psychosocial factors, we expect the strength of the association between ED and dependence to vary. Specifically, we would estimate a stronger link between ED and dependence among those countries with drier cultures as ED in those countries would be a stronger marker of psychosocial vulnerabilities that lead youth to drink beyond what is normative in that context; contrarily, we would expect a weaker association among the most permissive countries where ED is not outside the limits of acceptable drinking behavior. Given that countries' socioeconomic level could confound these associations (varying levels of early dependence detection or access to treatment, for instance), we will adjust by countries' socioeconomic level. Lastly, since the ED–dependence association might differ between genders (Tomek et al., 2016), we will characterize the association by gender.

MATERIALS AND METHODS

Design and Procedure

For this cross-sectional study, we used the global data available at the Global Information System on Alcohol and Health (GISAH) of the World Health Organization (2018a)[1]. Information of the GISAH measures comes from different sources, such as governmental reports, public statistics, local projects, and surveys and comprises more than 225 states (Poznyak et al., 2014). Here, data were available from 169 countries and missing from 25, which were, thus, excluded from the analyses. The gathered information included the following:

Early Drinking (ED): The percentage of those who were current alcohol drinkers between the ages of 15 and 19 years during the past 12 months, in each country.

Alcohol Dependence: The percentage of those (15 years or older) with a diagnosis of alcohol dependence according to the International Classification of Diseases (ICD) during the past 12 months, in each country.

Heavy Episodic Drinking (HED): The percentage of adults (15 years or older) who had at least 60 g of pure alcohol (approx. six standard alcoholic drinks) on at least one occasion in the last 30 days, in each country.

Actual Drinkers: The percentage of those who consumed any alcohol in the past 12 months, for each country.

Alcohol Policy: Whether the country adopted a written alcohol policy for reducing the burden on alcohol (yes/no/only at the subnational level/consumption prohibited).

We also used the following socioeconomic data from the World Bank (2019)[2].

Income Level: Classification of the World Bank (i.e., low, middle-low, upper-middle, and high) based on the national income per person, for each country.

Gini Index: A measure of how much the distribution of income deviates from perfect inequality (100) and absolute equality (0), in each country.

Data Analyses

To cluster countries by their shared drinking characteristics, we used, sequentially, two multivariate data reduction techniques:

[1]https://apps.who.int/gho/data/node.main.GISAH?lang=en
[2]https://data.worldbank.org/

a multiple correspondence analysis (MCA) and a hierarchic classification. MCA is a valuable tool for the description and visualization of relationships in complex categorical data without the need of distributional assumptions (Greenacre, 2017; Quail et al., 2017). For factor analysis, the active variables are those that contribute to the variance (inertia) in the data set and to the formation of factorial axes. We considered as active variables the following: actual drinking prevalence, heavy episodic drinking (HED) prevalence, and whether there is a written alcohol policy in place. Since written policy was a nominal variable, the first two variables (drinking and HED prevalences) were divided into quartiles; hence, 1 was the lowest prevalence and 4 the highest. Based on the factor analysis, the classification was carried out on the three main axes, and a partition was subsequentially performed.

To estimate the association between ED and alcohol dependence, we performed first descriptive analyses, and then, beta regressions (using logit link and maximum likelihood estimator) for all the countries together and also within each class. Beta regressions are a suitable approach for beta-distributed data, such as percentages, where a linear regression model is not accurate. In order to improve interpretation, we exponentiated the log odds of significant relationships to obtain the odds ratios. For regressions, the percentage of alcohol dependence was the outcome variable, and the percentage of ED was set as the predictor. The analyses were performed adjusting by income level and the Gini index, but because of multicollinearity among them, and the results being no different with either measure (not shown), only income level was used. These analyses were repeated by gender (i.e., percentage of alcohol dependence and early alcohol consumption in males and females). For each regression, we used diagnostic plots to assess the regression assumptions, i.e., no detectable patterns among Pearson's and deviance residuals (not shown). All regressions satisfied such conditions. Pseudo R^2 was obtained to estimate the goodness of fit.

For data management and analyses, the Système Portable pour l'Analyse de Données Numériques (SPAD-N) version 4.1 for Windows (Lebart et al., 1983) and the R software version 3.5.3 for Windows, package betareg (Zeileis et al., 2016), were used.

RESULTS

Clustering of Countries by Their Drinking Characteristics

Countries were clustered by their drinking characteristics (prevalence of HED and of actual drinkers, and alcohol policy) through factor analyses. The first three factorial axes were retained, accounting for 63.32% of the total variance. Hierarchic classification and partition resulted in four distinctive classes of drinking contexts and national policies. **Figure 1** shows the countries' pertinence to each cluster.

The first class (1/4; n = 33, 19.5%) clustered those countries with the lowest percentages of HED. Almost all of the countries in this class (96.9%) had very low percentages of actual drinkers. All the countries where consumption is prohibited were clustered here. This class seems to comprise those countries where abstention is the norm and could be considered *primarily abstainers*. The second class (2/4; n = 50, 29.6%) included those countries where HED was low; 97.6% of those with low HED prevalences and 97.5% of those with low drinking prevalences were clustered here. More than half of the countries in this class (62%) did not have a written alcohol policy. Countries in this class could be regarded as *low drinking* countries. The third class (3/4; n = 38, 22.5%) comprised those countries with high prevalences of HED (92% of those with HED on the third quartile were classified here) and of drinkers (89.5% of countries with a prevalence of drinkers on the third quartile were clustered here). More than half (66%) of the countries that had alcohol policies only at the subnational level were clustered in this class. These countries could be regarded as *high drinking* countries. The fourth class (4/4; n = 48, 28.4%) included those countries where the prevalence of HED was very high (91% of those countries were clustered here) and the prevalence of drinkers was also very high (89% of those countries were classified in this cluster). Most of the countries in this class (81%) had written alcohol policies. The countries in this class could be considered *very high drinking* countries (**Supplementary Table S1**).

Description of ED, Dependence, and Income Level in Each Country Cluster

An increase in ED was observed in classes 1–4, ranging from 5% in class 1 to almost 60% in class 4. An increase in alcohol dependence was also found, but not as marked as that of ED. Regarding income level, countries in classes 1 and 2 were mostly low or middle-low income, countries in class 3 were mostly middle-low or upper-middle, while class 4 was mainly composed of high-income-level countries. These results are presented in **Table 1**.

Relationships Between ED and Dependence for All Countries and by Country Cluster and Gender

The regression coefficients between ED and alcohol dependence for all countries and within each country cluster, unadjusted and adjusted by income level, are shown in **Table 2** and, for each gender, in **Table 3**. A positive relationship was found between ED and alcohol dependence for all the countries (OR = 5.58, adjusted by income OR = 7.46), class 1 (OR = 323.76, adjusted by income OR = 419.89), and class 2 (OR = 98.49, adjusted by income OR = 29.08). No significant relationships were found between ED and alcohol dependence in class 3 or in class 4. These results were similar for males, but not for females. For females in all the countries combined, a positive association was found (OR = 8.01, adjusted by income OR = 5.64), but no significant relationship was found in class 1; in class 2, a relationship was found only in unadjusted regressions (OR = 5767.53). For males in all countries combined, a positive association was found (OR = 4.35, adjusted by income OR = 6.29), in class 1 (OR = 78.26, adjusted by income OR = 88.23), and class 2 (OR = 16.61, adjusted by income OR = 8.25).

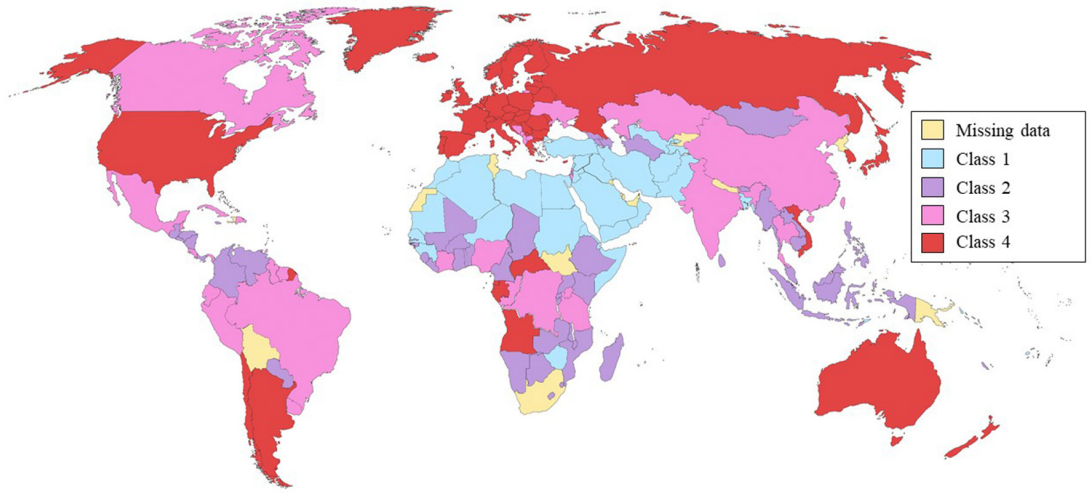

FIGURE 1 | Countries' clusters by actual drinking prevalence, heavy episodic drinking (HED) prevalence, and national alcohol policy.

DISCUSSION

In this article, we aimed to explore, first, whether there is an association between ED and dependence at the aggregate level as aggregate level data would not be as affected by selection bias as individual-level studies. Overall, this is for the world population, and for males and females separately, we found a moderate/strong association between ED and dependence. This finding is not surprising and concurs with other cross-sectional individual-level data. However, this evidence is not without divergence and so far has come almost exclusively from high-income countries (Maimaris and McCambridge, 2014; Kuntsche et al., 2016).

TABLE 1 | Early drinking (ED), alcohol dependence, and income level in each country class (by actual drinking prevalence, heavy episodic drinking prevalence, and national alcohol policy).

	n	Percentage	M (SD)
	Class 1 (n = 33)		
ED			4.75 (4.73)
Alcohol dependence			0.86 (1.01)
Income level			
Low	13	39	
Middle-low	13	39	
Upper-middle	4	12	
High	3	9	
	Class 2 (n = 50)		
ED			18.20 (4.25)
Alcohol dependence			2.04 (0.90)
Income level			
Low	22	44	
Middle-low	20	40	
Upper-middle	8	16	
High	–	–	
	Class 3 (n = 38)		
ED			34.89 (7.97)
Alcohol dependence			2.69 (0.89)
Income level			
Low	3	8	
Middle-low	10	26	
Upper-middle	18	47	
High	7	18	
	Class 4 (n = 48)		
ED			57.68 (11.45)
Alcohol dependence			3.7 (2.5)
Income level			
Low	2	4	
Middle-low	2	4	
Upper-middle	11	23	
High	33	69	

Clustering of Countries by Their Drinking Characteristics

In order to determine whether the association varied according to the drinking context, which we would expect if the association is due to psychosocial factors rather than biological, countries were clustered by their drinking characteristics. For that purpose, we applied, sequentially, two data reduction techniques that clustered countries by their percentage of actual drinkers, percentage of HED during the last month, and the national alcohol policy. We found four distinctive clusters of countries: two of them where abstention or infrequent drinking seems to be the norm and two where drinking is frequent. Notably, although socioeconomic data were not used for clustering, the first two classes were mostly formed by low- and middle-low-income countries, while the third was mainly by upper-middle-income countries and the fourth (depicting the highest level of drinking and HED) mostly by high-income countries. As expected, we found increasingly higher prevalences of ED and dependence in each of the four classes.

Relationships Between ED and Dependence for All Countries and by Country Cluster and Gender

Second, we explored whether the relationship between ED and dependence varied across countries clustered by their

TABLE 2 | Beta regression analyses for the relationship between alcohol dependence and early drinking (ED) for all countries and by each country class.

	Estimate (95% CI)	φ	R^2
	Total (n = 194)		
ED	1.72 (1.32–2.11)***	93.53***	0.29
ED[1]	2.01 (1.43–2.58)***	94.46***	0.31
	Class 1 (n = 33)		
ED	5.78 (0.59–10.98)*	149.49***	0.22
ED[1]	6.04 (0.39–11.69)*	149.83***	0.23
	Class 2 (n = 50)		
ED	4.59 (1.69–7.5)**	234.68***	0.15
ED[1]	3.37 (0.42–6.32)*	261.24***	0.24
	Class 3 (n = 38)		
ED	0.07 (−1.74–1.88)	170.4***	0.01
ED[1]	−0.83 (−2.75–1.08)	200.32***	0.11
	Class 4 (n = 48)		
ED	0.88 (−0.68–2.43)	61.52***	0.03
ED[1]	1.67 (−0.36–3.71)	63.14***	0.05

*The outcome variable was alcohol dependence in all the regressions. φ: Precision parameter. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. [1]Adjusted by country income level.*

shared drinking characteristics. We expected that, if the causal mechanism was of a biological nature, the association between ED and dependence would be fairly even among clusters of countries. Conversely, if the relationship was mainly due to psychosocial factors, we expected the strength of the association between ED and dependence to vary. Specifically, we estimated a stronger link between ED and dependence among those countries with drier cultures (by our results, primarily abstainers and low drinking classes) as ED in those countries would be a stronger marker of psychosocial vulnerabilities that lead youth to drinking beyond what is normative in that context; contrarily, we would expect a weaker association among the most permissive countries where ED is not outside the limits of acceptable drinking behavior (namely, high drinking and very high drinking classes). Our results confirmed this last hypothesis. We found positive and strong relationships between ED and alcohol dependence for the two clusters of countries where abstention or low infrequent drinking seems to be normative, while no significant relationships were found in those classes where drinking seems to be the common practice (the high drinking and very high drinking classes). Furthermore, the association was somewhat stronger for the primarily abstainers class (with higher abstention rates) than for the low drinking class. These results indicate that the association between ED and dependence varies according to the cultural context. This finding is supported by other authors (Maimaris and McCambridge, 2014; Kuntsche et al., 2016; Frøydis et al., 2019) who question the assumption that the association between ED and later problems is independent of cultural norms and national alcohol policies. Given that a solid association is a requirement for causation, our results would not lend empirical basis to a strong causal link between ED and dependence. Our findings suggest, at least at the epidemiological level, and as found in many of the few prospective studies that control for third variables (Aiken et al., 2018), that the association may be due to genetic, biologic, or psychosocial vulnerabilities that promote consumption outside cultural norms. However, since there is evidence of biological mechanisms that increase the risk of developing alcohol dependence after ED (Buchmann et al., 2009; Varlinskaya et al., 2020) and that ED may exacerbate genetic influences (Agrawal et al., 2009), an alternative possibility is that the causal link is anteceded by genetic psychosocial characteristics and also strongly moderated by them. That is, those with certain vulnerabilities (genetics for instance) may deviate from cultural norms and initiate early into consumption (marker hypothesis). ED may, in turn, put into place biological mechanisms which increase their risk of pathological consumption (causal link hypothesis), and depending on psychosocial and environmental characteristics, they may, or not, progress into dependence. Therefore, future research must imply large longitudinal studies that include different cultures and drinking contexts in order to assess the correspondence of changes in ED and alcohol-related problems over the years.

Given that countries' socioeconomic level could confound these associations (varying levels of ED detection or access to treatment, for instance), we adjusted by each country

TABLE 3 | Beta regression analyses for the relationship between alcohol dependence and early drinking (ED) for all countries and each country class by gender.

	Female			Male		
	Estimate (95% CI)	φ	R^2	Estimate (95% CI)	φ	R^2
			Total (n = 194)			
ED	2.08 (1.61–2.55)***	186.03***	0.29	1.47 (1.11–1.83)***	55.37***	0.29
ED[1]	1.73 (1.07–2.39)***	186***	0.31	1.84 (1.33–2.35)***	57.03***	0.33
			Class 1 (n = 33)			
ED	4.21 (−4.54–12.95)	605.3***	0.05	4.36 (0.67–8.05)*	77.37***	0.24
ED[1]	4.74 (−4.67–14.14)	607.4***	0.05	4.48 (0.39–11.69)*	77.43***	0.24
			Class 2 (n = 50)			
ED	8.66 (1.99–15.34)*	231.7***	0.11	2.81 (0.83–4.78)**	167.53***	0.13
ED[1]	5.59 (−1.05–12.22)	284.8***	0.27	2.1 (0.06–4.13)*	179.37***	0.19
			Class 3 (n = 38)			
ED	0.72 (−1.77–3.21)	194.6***	0.01	−0.3 (−1.88–1.27)	119.95***	0.01
ED[1]	−1.01 (−3.42–1.41)	286.59***	0.28	−0.91 (−2.65–0.82)	131.19***	0.07
			Class 4 (n = 48)			
ED	1.77 (0.33–3.20)	149.83***	0.18	0.33 (−1.29–1.96)	33.16***	0.01
ED[1]	1.55 (−0.19–3.31)	150.48***	0.18	1.44 (−0.87–3.75)	34.11***	0.03

*The outcome variable was alcohol dependence. φ: Precision parameter. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. [1]Adjusted by country income level.*

socioeconomic level. When adjusted by income, surprisingly, the association was stronger for the whole sample and for the primarily abstainers class, while it was attenuated for the low drinking class, suggesting that the country's socioeconomic level may be a moderator variable, by, for instance, facilitating or inhibiting the progression from drinking to dependence. Future studies should explore how societal socioeconomic conditions could affect drinking trajectories.

Lastly, since the ED–dependence association might differ between genders (Tomek et al., 2016), we characterized the association by gender. The results were similar for males, but not for females. For females in all the countries combined, a positive association was found (also attenuated when controlling for socioeconomic level), which indicated that, for each percentile increase in ED, there was almost a fivefold increase in the prevalence of dependence. However, we found no association between ED and dependence among females for any of the country clusters. Although consumption among women has increased over time, bearing in mind that we only found an association between ED and dependence among those country clusters where drinking is very infrequent, one possible rationale for this finding is that data for females in those countries were insufficient and that the analysis lacked statistical power. However, these findings suggest that, even if there was an association we could not detect with the available data, that association was weak. Conversely, the results for males were similar to those for the total sample, not surprisingly given that both consumption and dependence are higher among men (World Health Organization, 2018b), and thus, it is men's consumption which accounts for most of the whole sample variability.

The results presented here are not without limitations. The data we analyzed as ED were the prevalences of drinking on those aged 15–19 years in each country. First of all, these being aggregated cross-sectional data, we should bear in mind that those with ED are not the same individuals as those with alcohol dependence. Nonetheless, aggregated data have the advantage of not being affected by self-selection bias, a common limitation of longitudinal studies. Furthermore, some evidence signals that different ED measures (such as first sip, first drunkenness, or initiation of regular drinking) might yield different results (Morean et al., 2018). It has also been suggested (Maggs et al., 2019) that there might be differences in whether drinking initiation occurs during early adolescence or during childhood (i.e., before 11 years of age), and we did not consider those factors. However, research indicates (Connor et al., 2019) that the onset of regular drinking would be a more solid measure of later problems than others (such as age of first sip), giving support to the measure we used here.

To conclude, and despite limitations, we provide evidence that the ED–dependence association is not independent of cultural drinking norms and national alcohol policies. Furthermore, the results expose aspects that may add to the understanding of the relationship between ED and later problems. Such characteristics relate both to the individual and to the context in which alcohol is consumed, whether they are of a cultural nature such as gender, psychosocial such as social norms, or macroeconomic such as a country's income level.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed for this study. The datasets can be found here: World Bank Open Data, https://data.worldbank.org/ and here: Global Information System on Alcohol and Health (GISAH), https://apps.who.int/gho/data/node.main.GISAH?lang=en.

AUTHOR CONTRIBUTIONS

KC, RP, and MC contributed to the conception and design of the study. KC organized the database. KC and RP performed the statistical analysis. All authors wrote sections of the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

FUNDING

Partial funding was provided by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and UNMdP, Argentina.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00017/full#supplementary-material.

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 06 March 2020
doi: 10.3389/fnbeh.2020.00031

Effects of Ethanol Exposure During Adolescence or Adulthood on Locomotor Sensitization and Dopamine Levels in the Reward System

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: 08 August 2019
Accepted: 13 February 2020
Published: 06 March 2020

Citation:
Carrara-Nascimento PF, Hoffmann LB, Flório JC, Planeta CS and Camarini R (2020) Effects of Ethanol Exposure During Adolescence or Adulthood on Locomotor Sensitization and Dopamine Levels in the Reward System.
Front. Behav. Neurosci. 14:31.
doi: 10.3389/fnbeh.2020.00031

Behavioral sensitization is a process of neuroadaptation characterized by a gradual increase in motor behaviors. The major neural substrates involved in the behavioral sensitization lie on the dopaminergic mesocorticolimbic pathway, which is still under development during adolescence. To investigate age-differences in ethanol behavioral sensitization and dopamine levels in distinct brain regions of the reward system, adolescent and adult mice were repeatedly pretreated with saline or ethanol (2.0 g/kg i.p.) during 15 consecutive days and challenged with saline or ethanol 5 days after pretreatment. Dopamine and its metabolites were measured in tissue samples of the prefrontal cortex (PFC), nucleus accumbens (NAc) and striatum by HPLC analysis. While repeated ethanol administration resulted in the development of locomotor sensitization in both adult and adolescent mice, only the adults expressed sensitization to a subsequent ethanol challenge injection. Neurochemical results showed reduced dopamine levels in adolescents compared to adults. Specifically, mice pretreated with ethanol during adolescence displayed lower dopamine levels in the PFC compared to the respective adult group in response to an ethanol challenge injection, and preadolescent mice exhibited lower dopamine levels in the NAc following an acute ethanol treatment compared to adults. These findings suggest that adolescent mice are not only less sensitive to the expression of ethanol-induced sensitization than adults, but also show lower dopamine content after ethanol exposition in the PFC and NAc.

Keywords: ethanol, adolescence, prefrontal cortex, striatum, nucleus accumbens, mice

INTRODUCTION

Alcohol is a widely abused substance in human society, which is associated with economic, health and family costs. Adolescents who start drinking at the age of 14 or younger show a higher prevalence of lifetime alcohol use disorders than those who start drinking at ages 20 or older (Grant and Dawson, 1997), suggesting that early consumption of alcohol increases the vulnerability to addiction.

Adolescents show a characteristic pattern of behavioral responses to ethanol that differs from adults. Adolescent rodents are less sensitive to ethanol's sedative effects and to ethanol behavioral sensitization as compared to adults, but show higher sensitivity to its appetitive effects (Faria et al., 2008; Pautassi et al., 2008). The profile of neurochemical responses to ethanol in adolescents is also distinct from adults (Pascual et al., 2009; Guerri and Pascual, 2010; Carrara-Nascimento et al., 2011; Mishra and Chergui, 2013; Crews et al., 2016).

The protracted development of the mesocorticolimbic dopamine pathway explains, in part, typical characteristics of adolescents, such as cognition immaturity, impulsive behavior, novelty and reward-seeking and risky decision making (for review, see Spear, 2000). The prefrontal cortex (PFC) undergoes important developmental changes during adolescence in humans and rats (Insel et al., 1990; Giedd et al., 1999) and dopaminergic inputs to this region have inhibitory functions, enabling control of attention, motivation and decision making.

The limbic system undergoes important and determinant maturational changes for the transition from infancy to adulthood. Adolescent rats (at PND 30) exhibited lower basal levels of dopamine compared to adults in tissue samples of the striatum (Teicher et al., 1993) and reduced storage pool of releasable dopamine in this region (Stamford, 1989). Although similar dopamine basal levels were found in tissue samples of nucleus accumbens (NAc) and frontal cortex between adolescent and adult rats (Teicher et al., 1993), microdialysis studies demonstrated peaks at PND 45 compared to younger or older rats in the NAc (Badanich et al., 2006; Philpot et al., 2009). In general, basal dopamine efflux from NAc obey an inverted U-shaped curve (Philpot et al., 2009) and repeated ethanol exposure during adolescence alters the pattern of basal dopamine levels (Badanich et al., 2007; Pascual et al., 2009). These changes may be determinant to promote reward-seeking behavior (Pascual et al., 2009; Alaux-Cantin et al., 2013) since ethanol exposure during adolescence alters goal-directed behavior and judgment toward poor decision-making and risk-taking behavior (Goudriaan et al., 2007; see Alfonso-Loeches and Guerri, 2011, for review). In fact, stimulant effects of ethanol are determined mostly by its actions on the synthesis, release and turnover of dopamine of dopaminergic neurons (Fadda et al., 1980; Di Chiara and Imperato, 1985; Brodie et al., 1999; Bassareo et al., 2017), whose actions are responsible for its reinforcing effects.

Behavioral sensitization is a progressive increase in behavioral responses to drugs or stress that represents a neuroplastic outcome of enduring events occurring in the dopaminergic mesolimbic pathway (Wise and Bozarth, 1987; Robinson and Berridge, 2000). Behavioral sensitization is conceptualized into two phases: initiation and expression. The initiation or development of sensitization reflects the immediate events occurring in the ventral tegmental area (VTA), while the expression reveals long-term consequences of the initial neural alterations after cessation of the treatment (Kalivas and Stewart, 1991). In general, the neural changes underlying the expression of sensitization require a withdrawal period. Furthermore, there is evidence for the overlapping of neural circuitries responsible for behavioral sensitization and reinstatement (Steketee and Kalivas, 2011), suggesting a link between the expression of sensitization and relapse. Among the neural changes underlying behavioral sensitization, the mesocorticolimbic dopamine system is critically involved (Steketee and Kalivas, 2011). Studies focused on dopaminergic underpinnings of chronic ethanol consumption and withdrawal have shown that repeated and continuous exposure to ethanol followed by withdrawal periods results most often in a hypodopaminergic state (Diana et al., 1996), although the hyperdopaminergic state has been reported in protracted abstinence (Hirth et al., 2016). An electrophysiological study showed that ethanol-induced behavioral sensitization induced enhancement of the basal spontaneous firing rate of dopamine neurons in the VTA (Didone et al., 2016).

A different profile of cocaine behavioral sensitization and dopaminergic neurochemical sensitization were found in adolescent mice compared to their adult counterparts. Adolescents exhibited greater behavioral sensitization and lower sensitization to dopamine overflow compared to adults (Camarini et al., 2008), which was associated with a higher expectancy of the drug in adolescents. However, contrary to what was observed with cocaine (Camarini et al., 2008; Valzachi et al., 2013), adolescent mice are less sensitive to ethanol-induced locomotor sensitization than adults (Stevenson et al., 2008; Carrara-Nascimento et al., 2011; Camarini and Pautassi, 2016). Despite the existing literature on alcohol-induced changes in the dopaminergic system of adolescents, studies do not correlate ethanol behavioral sensitization with alterations in dopamine levels in brain regions involved in the phenomenon.

The present study aimed to evaluate locomotor behavioral responses to acute and repeated ethanol in adolescent and adult mice and quantify their dopamine and its metabolites levels in tissue homogenates of specific brain regions related to the reward system (PFC, striatum, and NAc).

MATERIALS AND METHODS

Animals

Adolescent and adult male Swiss mice were obtained from the Animal Facility of the Department of Pharmacology of the Institute of Biomedical Sciences at the Universidade de São Paulo, Brazil. Mice were housed in groups of five in standard Plexiglas cages (30 cm × 20 cm × 12.5 cm) in a colony room with controlled lighting (12:12 light/dark cycle; lights on from 7:00 AM to 7:00 PM) and temperature (22 ± 2°C) conditions. All mice were allowed to adapt to the colony room for at least 7 days before the beginning of the experiments. At the beginning of the experiments, adolescents were PND 28–30 and adults, PND 68–70. Food and water were provided *ad libitum*. All procedures were approved by the Ethics Committee on Animal Use (Comitê de Ética no Uso de Animais—CEUA—Protocol #81/2013) of the Institute of Biomedical Sciences of the Universidade de São Paulo.

Drugs

Ethanol (Merck do Brasil, Rio de Janeiro, RJ, Brazil) solution at 20% was prepared from 95% (v/v) ethanol and administered at a dose of 2.0 g/kg *via* intraperitoneal injections. This dose was achieved by administering 0.125 ml per 10 g of body weight. Control mice were administered isovolumetric injections of the vehicle solution (0.9% v/v saline). The dose and treatment regimen were chosen based on previous studies that show reliable behavioral sensitization in Swiss male mice (for review, see Camarini and Pautassi, 2016). For instance, initiation and expression of sensitization depend on a number of factors: number of injections and interval between them, ethanol dose, species, strain, sex, among others. In Didone et al. (2008), lower ethanol doses (1.5–2.0 g/kg) resulted in better expression during the first 10–15 min after ethanol injection than higher doses (2.5–3.0 g/kg).

Apparatus

The locomotor activity was assessed in a cylindrical open-field arena (40 cm diameter and 35 cm high). A video camera, placed above the apparatus and connected to a computer located outside the experimental room, recorded the trials. The apparatus was cleaned with a 5% ethanol/water solution between each trial. Injections and locomotor activity assessments were always carried out between 9:00 AM and 11:30 AM.

Behavioral Sensitization Procedure

First, animals were habituated to the injections and open-field apparatus for two consecutive days (Habituation days: H1 and H2). Mice were injected intraperitoneally (i.p.) with saline (0.9% w/v sodium chloride, SAL) and placed in the open-field for 5 min, 5 min after the injection.

The experimental design of the behavioral sensitization consisted of a phase of initiation of locomotor sensitization (15 days), followed by an abstinence period (5 days) and then, by a test day, when the expression of sensitization was evaluated.

On the next day after the last habituation session, adolescent and adult mice were distributed into the experimental groups (saline and ethanol) for the initiation of behavioral sensitization. One-half of mice of each age group received daily i.p. injections of saline, while the other half was treated with 2.0 g/kg ethanol (20% v/v ethanol in saline), resulting in four experimental groups: Adolescent-SAL (n = 20), Adolescent-EtOH (n = 20), Adult-SAL (n = 20) and Adult-EtOH (n = 20). The treatment lasted 15 days and the locomotor activity was quantified only on days 1, 8 and 15. Animals were exposed to the open-field arena only during recording days, as previously described in Camarini et al. (2008). The animals' locomotor activity (distance traveled in cm) was assessed during a 5 min-period, 5 min after saline or ethanol injection. This period (5–10 min after injection) fits in the time window of the peak of the acute stimulation and the locomotor sensitization effect of ethanol (Phillips et al., 1995; Legastelois et al., 2015), Moreover, this procedure minimizes any association of discomfort due to ethanol injection with the apparatus.

After 5 days of abstinence, on experimental day 21, mice were tested for the expression of ethanol sensitization. Half of each experimental group was challenged with 2.0 g/kg ethanol, while the other half was injected with a saline injection, establishing eight experimental groups: Adolescent-SAL/SAL (n = 10), Adolescent-SAL/EtOH (n = 10), Adolescent-EtOH/SAL (n = 10), Adolescent-EtOH/EtOH (n = 10), Adult-SAL/SAL (n = 10), Adult-SAL/EtOH (n = 10), Adult-EtOH/SAL (n = 10) and Adult-EtOH/EtOH (n = 10). The expression of sensitization was conducted in the adolescent group on PND 50–52, and in the adult group on PND 90–92.

Mice were euthanized by cervical dislocation 40 min after the injections since peaks of striatal extracellular dopamine after systemic injection of 2.0 g/kg ethanol is reached around 40 min (Bosse and Mathews, 2011).

Quantification of Dopamine and Metabolites in the Brain Tissue

The brains were removed, cooled on ice, and three brain regions were dissected, based on the mouse brain atlas (Paxinos and Franklin, 2001). Brains were placed in a mouse brain matrix (ASI-Instruments®, Houston, TX, USA), used to provide coronal brain sections. The brains were cut and mounted on slides (SuperFrost Plus, Thermo Fisher Scientific, MA, USA). Brain punches (1.2 mm or 1.0 mm) of the PFC, NAc, and striatum were obtained with micro punches (Harris Micro-Punch, Ted Pella). Specifically, the punched area in the frontal cortex was focused in the mPFC. The brain tissues were frozen in liquid nitrogen and maintained at −80°C for later quantification of dopamine and the metabolites DOPAC (3,4-Dihydroxyphenylacetic acid) and HVA (homovanillic acid).

The tissues (PFC, NAc and striatum) were homogenized and sonicated in 0.1 M perchloric acid solution, prepared by adding 8.68 mL of concentrated perchloric acid, 200 mg of sodium metabisulphite—$Na_2S_2O_5$—and 200 mg of EDTA in 1.0 L of MilliQ ultrapure water, containing 28.9 ng/mL of dihydroxybenzylamine (DHBA). The homogenates were centrifuged at 10,000 rpm for 20 min at 4°C. At the time of homogenization, the tissues were weighed (still frozen) immediately before adding the perchloric acid solution. For each mg of tissue, 15 μl of the perchloric acid solution with DHBA was added. Dopamine, DOPAC and HVA were measured by high-performance liquid chromatography with an electrochemical detector (HPLC model LC20 AD, Shimadzu, Japan and Detector Antec Decade sdc VT 03 electrochemical Flow Cell), with a C-18 column (Shimpak; ODS, 15 cm, Kyoto, Japan), and an integrator (model 20AC Chromatopac; Shimadzu). The limit of detection was 0.02 ng for DA, DOPAC and HVA.

Statistical Analysis

Details of the statistical test and sample size for each experiment are summarized in **Supplementary Table S1**.

The locomotor activity evaluated throughout the days (habituation days, H1 and H2 and treatment days, D1, D8 and D15) was analyzed by three-way ANOVAs, considering three factors [Age (adolescent and adult)] × [Treatment (saline or ethanol)] × Days as repeated measures. This analysis allowed to compare significant differences between adolescents and adults.

When appropriate, two-way ANOVAs were followed-up to analyze differences within each age, considering treatment and days as repeated measures. Data from the locomotor activity measured on the challenge day, when mice pretreated with saline or ethanol were challenged with saline or ethanol (Challenge Day), were analyzed by a three-way ANOVA, considering three factors [Age (adolescent and adult)] × [Pretreatment (saline or ethanol)] × [Challenge injection (saline or ethanol)]. Two-way ANOVAs [Pretreatment (saline or ethanol)] × [Challenge injection (saline or ethanol)] were conducted for each age group.

Dopamine and metabolites levels were analyzed by a three-way ANOVA considering three factors [Age (adolescent and adult)] × [Pretreatment (saline or ethanol)] × [Challenge injection (saline or ethanol)]. Two-way ANOVAs [Pretreatment (saline or ethanol)] × [Challenge injection (saline or ethanol)] were conducted for each age group.

ANOVAs were followed by Tukey HSD test as *post hoc* when significant interactions of factors were detected. A Bonferroni-corrected multiple comparison test was used when only significant main effects were found.

Levene's test was employed to test homogeneity of variance and assumptions for normal distribution was tested with the Shapiro–Wilks test. In case the analyses were found not normally distributed or due to unequal variance, Kruskal–Wallis was used to assess differences among the groups. Mann–Whitney test was used for pairwise comparisons.

The data are presented as mean ± SEM, except for the data from PFC (non-parametric data), which are expressed as the median values and interquartile range. Statistical significance was considered when $p < 0.05$. The program SPSS Statistics for Windows was used to analyze the data (SPSS Statistics, Armonk, NY, USA: IBM Corporation, Armonk, NY, USA).

RESULTS

Behavioral Sensitization

Habituation Days

Analysis of the data by a three-way ANOVA revealed a main effect of days (day effect, $p < 0.001$; **Figure 1A**). Bonferroni-corrected comparisons indicated that the locomotor activity of each group on H2 (second day of habituation) was significantly reduced compared to that on H1 (first day of habituation), consistent with habituation to the apparatus.

Development of Ethanol Behavioral Sensitization

Analysis of the data by a three-way ANOVA revealed main effects of treatment ($p < 0.001$), days ($p < 0.001$) and a treatment × days interaction ($p < 0.001$; **Figure 1A**). Main effect of age was non-significant ($p = 0.82$). Two-way ANOVAs were applied separately to the locomotor activity data from each age. Analysis of the adolescent data revealed main effects of treatment ($p < 0.001$), days ($p < 0.001$) and a treatment × days interaction ($p < 0.001$; **Figure 1A**). Tukey HSD test revealed that adolescent mice treated with ethanol exhibited higher locomotion than saline counterparts on days 8 and 15). The locomotion of ethanol-treated mice was greater on days 8 and 15 compared to day 1 (first session). There were no significant differences in the locomotor activity of mice treated with saline. Analysis of the adult data revealed main effects of treatment ($p < 0.001$), days ($p < 0.001$) and a treatment × days interaction ($p < 0.001$; **Figure 1A**). Tukey HSD test revealed similar results as those observed in the adolescent group.

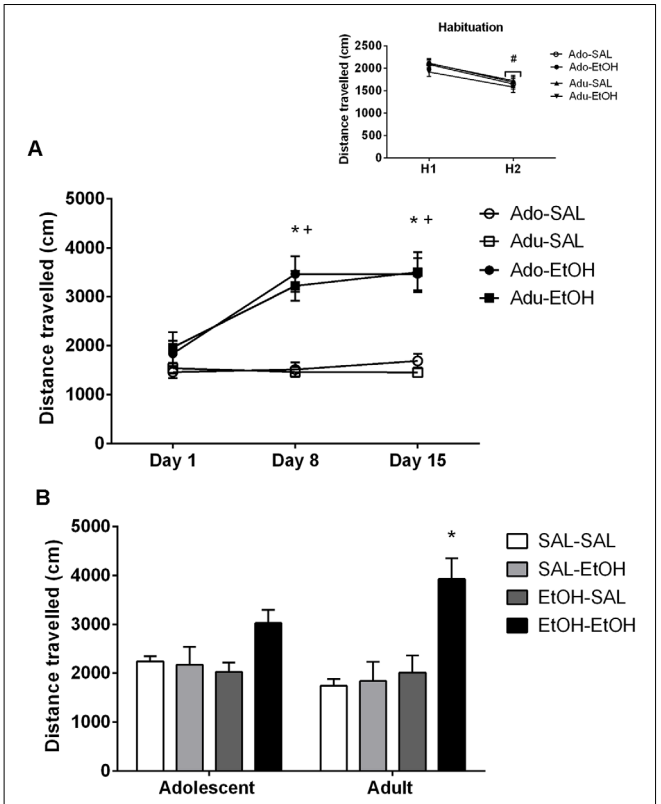

FIGURE 1 | Locomotor activity of adolescent and adult mice. **(A)** Adolescent (Ado) and adult (Adu) mice were pretreated with saline (SAL) or ethanol (EtOH) during 15 consecutive days and the locomotor activity was assessed on Days 1, 8 and 15. *Denotes significant differences in locomotor activity from Day 1; $^{+}$denotes significant differences in locomotor activity of ethanol-treated mice compared to saline-treated mice. The smaller figure shows the locomotion of mice injected with saline during two consecutive days (habituation days), $^{\#}$locomotion of each group on the second day of habituation (H2) was lower than on the first day they were exposed to the open-field (habituation day: H1). **(B)** Locomotor activity assessed on Day 21 in mice challenged with saline (-SAL) or ethanol (-EtOH) 5 days after pretreatment with saline (SAL-) or ethanol (EtOH-). *Denotes greater locomotor activity compared to all the other groups within the same age group. The values are expressed as mean ± SEM.

Expression of Ethanol Behavioral Sensitization

The locomotion of mice pretreated with saline or ethanol and challenged with saline or ethanol is depicted in **Figure 1B**. Analysis of the data by a three-way ANOVA revealed main effects of pretreatment ($p < 0.01$), challenge injection ($p < 0.01$) and a pretreatment × challenge injection interaction ($p < 0.01$; **Figure 1B**). Main effect of age was non-significant ($p = 0.94$). Tukey HSD test performed to analyze pretreatment × challenge injection interaction revealed that mice pretreated with ethanol and challenged with ethanol (EtOH-EtOH) displayed greater locomotor activity than those mice challenged with saline

(EtOH-SAL). Two-way ANOVAs were followed-up to analyze differences within each age group. Analysis of the adolescent data revealed a pretreatment × challenge injection interaction ($p < 0.05$). Tukey HSD test did not reveal significant differences among adolescent groups, except for a trend between EtOH-SAL and EtOH-EtOH ($p = 0.051$). Analysis of the adult data revealed a pretreatment effect ($p < 0.01$), a main effect of challenge injection ($p < 0.01$) and a pretreatment × challenge injection interaction ($p < 0.05$). Tukey HSD test revealed that adult mice pretreated and challenged with ethanol (EtOH-EtOH) displayed greater locomotor activity compared to all the other groups (all p's < 0.001), suggesting a robust expression of behavioral sensitization.

Dopamine and Metabolites Quantification

Dopamine and metabolites were quantified in mice at PND 50–52 (Adolescent group) and at PND 90–92 (Adult group).

Prefrontal Cortex

The results of DA, DOPAC and HVA levels are depicted in **Figure 2**. Number of samples/group = 10. The non-parametric Kruskal–Wallis test was used for the analysis of dopamine and metabolites, and Mann–Whitney's test was used to assess the differences between experimental groups.

Dopamine

Pair-wise comparisons revealed that dopamine levels were lower in adolescent mice pretreated and challenged with ethanol (EtOH-EtOH) compared to the respective adult group ($U = 24$, $Z = -1.97$, $p < 0.05$) and to the adolescent mice pretreated with saline and challenged with ethanol ($U = 5$; $Z = 3.4$, $p < 0.05$). Adult mice pretreated and challenged with ethanol (EtOH-EtOH) exhibited higher dopamine levels than those pretreated with ethanol and challenged with saline (EtOH-SAL; $U = 24$, $Z = -1.9$; $p < 0.05$).

DOPAC

Pair-wise comparisons revealed that DOPAC levels were lower in adolescent mice pretreated and challenged with ethanol (EtOH-EtOH) compared to the respective adult group ($U = 24$, $Z = -2.00$, $p < 0.05$). Adult mice pretreated and challenged with ethanol (EtOH-EtOH) exhibited higher DOPAC levels than those pretreated with ethanol and challenged with saline (EtOH-SAL; $U = 22$, $Z = -2.12$; $p < 0.05$).

HVA

Pair-wise comparisons revealed that HVA levels were lower in adolescent mice pretreated and challenged with ethanol (EtOH-EtOH) compared to the respective adult group ($U = 12$, $Z = -2.87$, $p < 0.001$). Adolescent mice pretreated with saline and challenged with ethanol (SAL-EtOH) displayed higher HVA levels compared to controls (SAL-SAL; $U = 10$, $Z = -3.02$; $p < 0.001$) and to mice repeatedly treated with ethanol and challenged with ethanol (EtOH-EtOH; $U = 5$, $Z = 3.4$, $p < 0.001$).

Nucleus Accumbens

The results are shown in **Figure 3**. Number of samples/group = 8. Few samples were lost because of analytical failure.

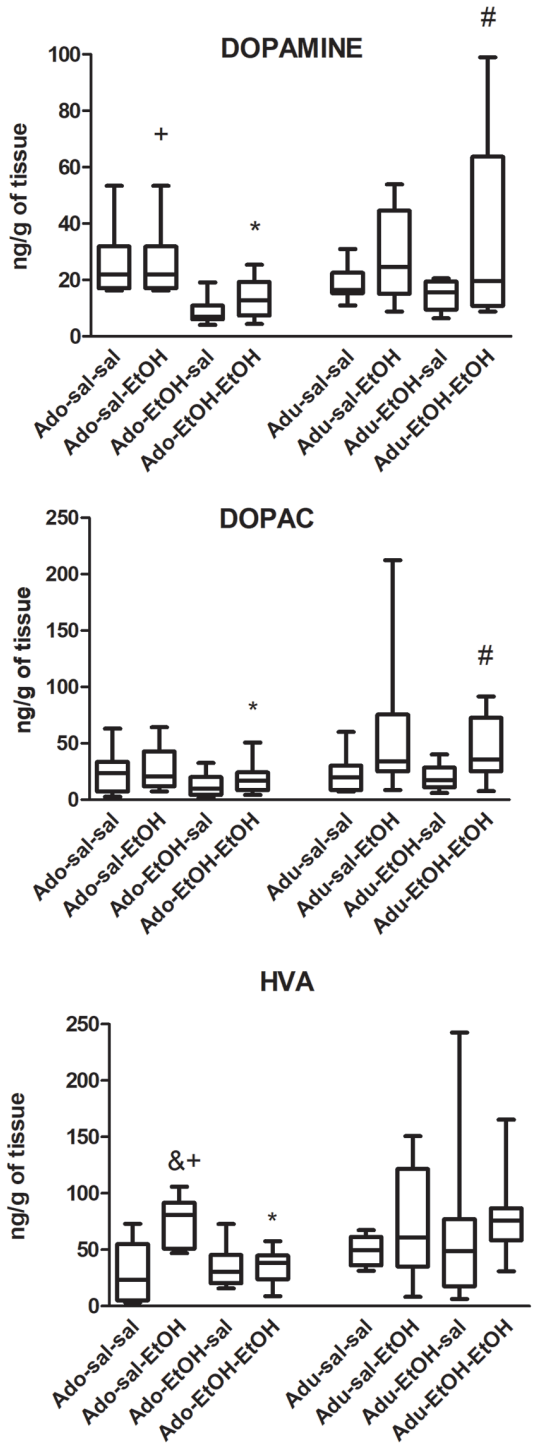

FIGURE 2 | Dopamine, DOPAC and HVA levels in the prefrontal cortex (PFC) of adolescent and adult mice pretreated with saline (SAL-) or ethanol (EtOH-) and challenged with saline (-SAL) or ethanol (-EtOH). *Adolescent group differ from the respective adult group, within the same treatment group. #Different from mice pretreated with ethanol and challenged with saline (EtOH-SAL) within the same age group, +differ from mice pretreated and challenged with ethanol (EtOH-EtOH), within the same age group, &different from the respective control group, within the same age group (SAL-SAL). Data are expressed as median and interquartile range.

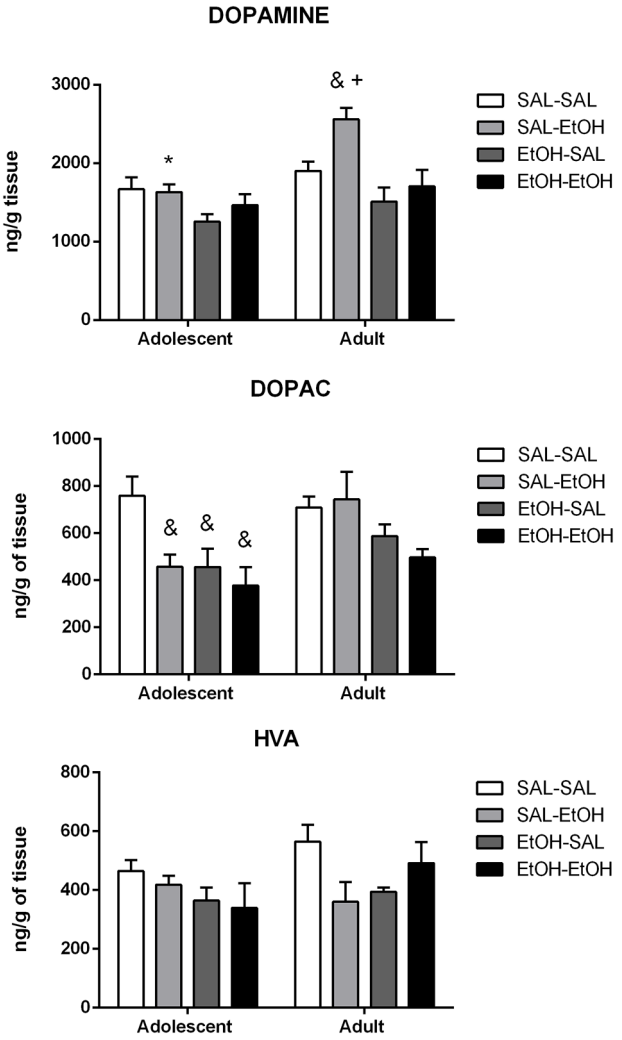

FIGURE 3 | Dopamine, DOPAC and HVA levels in the nucleus accumbens (NAc) of adolescent and adult mice pretreated with saline (SAL-) or ethanol (EtOH-) and challenged with saline (-SAL) or ethanol (-EtOH). *Adolescent mice differ from the respective adult mice, within the same treatment group, $^{+}$different from ethanol-pretreated mice challenged with ethanol (EtOH-EtOH), within the same age group, $^{\&}$different from the respective control group, within the same age group (SAL-SAL). The values are expressed as mean ± SEM.

Dopamine

Analysis of the data by a three-way ANOVA revealed main effects of age ($p < 0.001$), pretreatment ($p < 0.001$) and challenge injection ($p < 0.01$). Multiple comparisons with Bonferroni correction showed that the difference was between the age groups repeatedly treated with saline and challenged with ethanol (SAL-EtOH).

Two-way ANOVAs were used to analyze differences within each age. Although ANOVA has revealed the main effect of pretreatment ($p < 0.05$), *post hoc* pairwise comparisons did not show significant differences among adolescent groups. Analysis of the adult data by a two-way ANOVA revealed main effects of pretreatment ($p < 0.001$) and challenge injection ($p < 0.05$). Multiple comparisons with Bonferroni correction indicated that mice pretreated with saline and challenged with ethanol (SAL-EtOH) exhibited higher dopamine levels compared to their control group (SAL-SAL) and to mice repeatedly treated with ethanol and challenged with ethanol (EtOH-EtOH).

DOPAC

Analysis of the data by a three-way ANOVA revealed main effects of age ($p < 0.05$), pretreatment ($p < 0.001$) and challenge injection ($p < 0.05$). However, planned pairwise comparisons with Bonferroni correction did not show significant differences between age groups.

Two-way ANOVAs were used to analyze differences within each age. Analysis of the adolescent data by a two-way ANOVA revealed main effects of pretreatment ($p < 0.05$) and challenge injection ($p < 0.05$). Multiple comparisons with Bonferroni correction revealed lower DOPAC levels in mice treated with acute ethanol (SAL-EtOH) or pretreated with ethanol (EtOH-SAL or EtOH-EtOH) compared to their control group (SAL-SAL). Two-way ANOVA performed on the adult data revealed a main effect of pretreatment ($p < 0.05$). However, pairwise comparisons with Bonferroni correction did not show significant differences between age groups.

HVA

Analysis of the data by a three-way ANOVA did not find statistically significant differences between adolescent and adult groups (age effect, $p > 0.05$).

Two-way ANOVAs were used to analyze differences within each age. No statistically significant differences were found among groups for adolescents or for adults.

Striatum

The results are shown in **Figure 4**. Number of samples/group = 10.

Dopamine

Analysis of the data by a three-way ANOVA did not find statistically significant differences between adolescent and adult groups (age effect, $p > 0.05$).

Two-way ANOVAs were used to analyze differences within each age. No statistically significant differences were found among adolescent groups. Two-way ANOVA performed on the adult data revealed a main effect of pretreatment ($p < 0.05$). However, pairwise comparisons revealed only a statistically non-significant trend to increased dopamine levels in ethanol-pretreated adult mice challenged with ethanol compared to their controls (SAL-SAL; $p = 0.06$).

DOPAC

Analysis of the data by a three-way ANOVA did not find statistically significant differences between adolescent and adult groups (age effect, $p > 0.05$).

Two-way ANOVAs were used to analyze differences within each age. Analysis of the adolescent data by a two-way ANOVA revealed a main effect of pretreatment ($p < 0.001$). Pairwise comparisons with Bonferroni correction revealed increased DOPAC levels in ethanol-pretreated adolescent mice challenged with saline (EtOH-SAL) or ethanol (EtOH-EtOH)

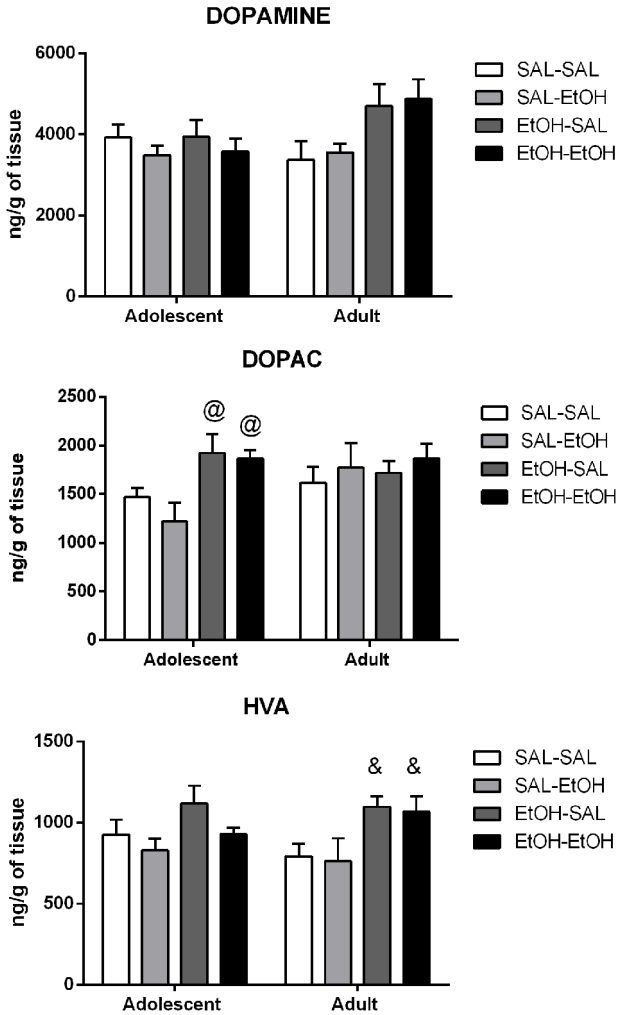

FIGURE 4 | Dopamine, DOPAC and HVA levels in the striatum of adolescent and adult mice pretreated with saline (SAL-) or ethanol (EtOH-) and challenged with saline (-SAL) or ethanol (-EtOH). [@]Different from the saline-pretreated mice challenged with ethanol, within the same age group (SAL-EtOH), [&]different from the respective control group, within the same age group (SAL-SAL). The values are expressed as mean ± SEM.

compared to those mice acutely treated with ethanol (SAL-EtOH). No statistically significant differences were found among adult groups.

HVA Levels

Analysis of the data by a three-way ANOVA did not find statistically significant differences between adolescent and adult groups (age effect, $p > 0.05$).

Two-way ANOVAs were used to analyze differences within each age. No statistically significant differences were found among adolescent groups. Two-way ANOVA performed on the adult data revealed a main effect of pretreatment ($p < 0.01$). Pairwise comparisons with Bonferroni correction revealed that ethanol-pretreated adult mice challenged with saline (EtOH-SAL) or ethanol (EtOH-EtOH) displayed higher HVA levels than their control group (SAL-SAL).

DISCUSSION

The present study suggests that adolescent mice are less sensitive to the expression of locomotor sensitization to ethanol as compared to adults, which has been previously demonstrated by other studies (Faria et al., 2008; Stevenson et al., 2008; Quoilin et al., 2012; Soares-Simi et al., 2013; Carrara-Nascimento et al., 2014, 2017) and highlights differential levels of dopamine in brain regions involved in the rewarding circuitry.

The attenuated effect on sensitization expression in adolescents could be admitted to a possible ceiling effect in response to 2.0 g/kg ethanol. However, this is unlikely because mice can sensitize to higher doses of ethanol, like 2.5 or 4.0 g/kg (Stevenson et al., 2008; Quoilin et al., 2012). We also questioned if ethanol exposure during adolescence could result in metabolic changes and alter the pharmacological profile to ethanol responses. Although this possibility cannot be discarded, previous studies have investigated the consequences of ethanol exposure during adolescence on blood alcohol concentration (BEC) and aldehyde dehydrogenase activity (ALDH). No differences in BECs were found between adolescents and adults following repeated ethanol administration, despite the differential magnitude in behavioral sensitization between them (Stevenson et al., 2008; Quoilin et al., 2012). Also, chronic ethanol pretreatment in adolescent and adult mice did not result in differential ALDH activity, although it had an impact on ethanol consumption patterns (Carrara-Nascimento et al., 2017). Despite these pieces of evidence, a study conducted by Linsenbardt et al. (2009) demonstrated that adolescent mice exhibited lower BEC than adults after acute and chronic administration of 4.0 g/kg ethanol.

Age-dependent differences in behavioral responses are not limited to alcohol. For instance, adolescent rats exhibited lower sensitivity to kappa agonists compared to adults (Anderson et al., 2013). Interestingly, ethanol and opioids share mechanisms of action to increase dopamine release (Lindholm et al., 2007).

Besides the reduced sensitivity to expression of ethanol sensitization, the main findings of the dopamine analysis in homogenates of brain regions suggest lower content after ethanol exposition in the PFC, and NAc of adolescents compared to adults. Although it is reasonable to speculate that adolescents have achieved their ceiling effect with 2.0 g/kg ethanol dose, our study was conducted in tissue samples instead of dialysates, and it has been reported that 2.0 g/kg ethanol enhances extracellular levels of dopamine by only 40% from the basal levels (Yim et al., 2000).

Acute ethanol has direct effects on dopaminergic neurons at VTA, which can alter dopamine release and its activity in the PFC (Harrison et al., 2017). Previous studies have demonstrated increased dopamine levels in the PFC after acute i.v infusion or posterior VTA administration of ethanol in adult rats (Ding et al., 2011; Schier et al., 2013). In the present study, we did not find significant increases in dopamine levels after acute ethanol in adolescent or adult mice, albeit dopamine and DOPAC levels were elevated in adult mice

repeatedly treated with ethanol following a challenge ethanol injection compared to the respective adult group challenged with saline. These results are particularly interesting because of the contribution of dopaminergic neurotransmission in the PFC to behavioral sensitization (Bjijou et al., 2002). Moreover, adolescent mice repeatedly treated and challenged with ethanol exhibited lower dopamine levels in the PFC compared to the respective adult group and to adolescent mice that received an acute ethanol injection. Similar age differences were found for DOPAC and HVA results. The findings also suggest the development of a dopaminergic tolerance to repeated ethanol treatment in the adolescent group. The PFC receives dopaminergic projections into the prelimbic and infralimbic regions that are involved in goal-directed behaviors (Hitchcott et al., 2007), cognitive control processes, motivation, and in responses to salient and relevant stimuli (Ott and Nieder, 2019). We have previously demonstrated that repeated exposure to ethanol during adolescence lowered Fos and Egr-1 protein expression (Faria et al., 2008) and cAMP response element-binding protein (CREB)-binding activity in the PFC (Soares-Simi et al., 2013) compared to adults. The dopamine D_1 signaling activation initiates a cascade of molecular events that modify transcription factors activity and gene expression, such as CREB, *c-fos, egr-1* (Nestler, 2001). Altogether, the present findings (reduced dopamine levels in adolescents treated with repeated ethanol) combined with previous studies (Faria et al., 2008; Soares-Simi et al., 2013) suggest a down-regulation of dopamine signaling mediated by D_1 receptors in those mice. In other words, ethanol exposure during adolescence blunts the D1-CREB-cFos signaling stimulated by repeated ethanol. Moreover, Pascual et al. (2009) found a decreased expression of D_1 receptors in the PFC of adolescent rats repeatedly treated with ethanol. However, we cannot discard the hypothesis that the dopamine results might be related to presynaptic effects, since dopamine D_2 has a key role in the synthesis, release and reuptake of dopamine. Thus, both effects can co-exist to further show a decrease in dopamine signaling. In sum, different neuroplasticity pattern in the PFC could contribute to the variability in the behavioral sensitization to ethanol in adolescents, considering the role of this brain region in the phenomenon (Li et al., 1993). It is important to emphasize that the assumptions on the dopamine system signaling are limited by the fact that the analyses were performed *ex vivo*.

In addition, the low PFC dopamine content in ethanol-pretreated adult mice challenged with saline (EtOH-SAL) most likely reflects the response of a withdrawal state, reversed by an ethanol challenge injection (EtOH-EtOH).

The present results also demonstrated a main effect of age for dopamine levels in the NAc, with adolescents acutely treated with ethanol exhibiting lower levels of dopamine compared to the respective adult group. Acute exposure to ethanol (SAL-EtOH) resulted in increased NAc dopamine content in adult mice compared to their controls (SAL-SAL), which is in agreement with other studies (Di Chiara and Imperato, 1985; Peters et al., 2017). However, this effect was not evident in adolescent mice. Other studies have found decreased evoked dopamine release in the NAc of rats treated with ethanol during adolescence (Philpot et al., 2009; Zandy et al., 2015; Shnitko et al., 2016). It is important to address that these past investigations reported low ethanol-stimulated dopamine responses in distinct periods of adolescence or young adulthood. Philpot et al. (2009) detected these differences in pre and early adolescence, while Zandy et al. (2015) and Shnitko et al. (2016) treated the rats during adolescence and measured ethanol-evoked dopamine efflux during their adulthood.

Since ethanol-pretreated mice (EtOH-EtOH) showed lower Nac dopamine levels than those pretreated with saline (SAL-EtOH) in response to an ethanol challenge injection, one could suggest a dopaminergic tolerance to repeated ethanol in adults in this region. It is important to emphasize, though, that the lower dopamine responses to ethanol in the NAc may reflect a response to a withdrawal effect that was not reversed by an ethanol challenge. Indeed, reduced dopamine outflow in the NAc after withdrawal has also been previously reported (Diana et al., 1993; Schulteis et al., 1995; Karkhanis et al., 2015). Furthermore, the opposite dopaminergic responses to repeated ethanol in the PFC vs. NAc in adult mice is consistent with the evidence of the inhibitory influence of PFC on mesolimbic dopaminergic transmission (Banks and Gratton, 1995).

Compared to the PFC and NAc, striatal dopamine levels were less affected by age-dependent factors. Repeated ethanol treatment showed a trend to enhance dopamine levels in adults but not adolescents. Although behavioral sensitization is not necessarily dependent on enhanced dopamine release in the striatum (Segal and Kuczenski, 1992), the expression of sensitization reflects, at least in part, neuroadaptations in the nigrostriatal dopaminergic pathway (Kalivas and Stewart, 1991). Thus, elevated dopamine striatal levels in adults repeatedly treated with ethanol can be, at least in part, responsible for their higher sensitivity to express ethanol-induced behavioral sensitization compared to adolescents (Faria et al., 2008; Stevenson et al., 2008; Carrara-Nascimento et al., 2014; Carrara-Nascimento et al., 2017).

We chose to evaluate dopamine levels in the PFC, NAc, and striatum because of the implication of these brain regions in addictive behaviors, such as behavioral inhibitory control, motivation, drug-related hedonic effects, habit formation, and behavioral sensitization (Koob and Bloom, 1988; White, 1996; Volkow and Fowler, 2000; Berridge and Robinson, 2016). Moreover, the PFC is still under maturation during adolescence and the frontal dopaminergic system has crucial importance in motivated behaviors. Dysfunctions of PFC have been associated with impaired inhibition to self-administer a drug (Volkow et al., 2002). Our findings provide evidence that dopaminergic responses to ethanol exposure during adolescence were less intense than that induced by ethanol exposure in adults.

Drugs of abuse have the ability to disrupt the dopaminergic system and promote an unstable and dynamic state of dopamine activity, depending on the recurrent process of addiction, i.e., intoxication, withdrawal or relapse. Our data showed age-dependent differences in dopaminergic responses mainly in the PFC and NAc, with few alterations in the

striatum. These regional differences may be attributed to the late ontogenic development of the PFC (for review, see Spear, 2000). NAc, in its turn, receives inputs from the PFC (Pennartz et al., 1994).

Although this study provided important age-dependent changes in the expression of behavioral sensitization and in brain regional dopamine responses to acute and repeated ethanol treatment, the neurochemical analysis was carried out 30 min after the behavioral test. This discrepancy might have implications in the direct correlations between neurochemical and behavioral effects.

A limitation of our study is that the dopamine analyses were conducted in tissue homogenates, which may not reflect a transient dopaminergic response. Although microdialysis would be more appropriate to monitor extracellular levels of dopamine over time, we aimed to investigate whether ethanol pretreatment during adolescence would change the behavioral response to ethanol and produce differences in DA levels in distinct brain regions of the reward system as a result of the lasting effects of ethanol exposure during the brain development. Despite the limitation, the age differences in dopamine and its metabolites promoted by acute or repeated ethanol in those brain regions reflect disturbances in numerous factors that can be related to synthesis, release, uptake, and metabolism of dopamine. Those results contribute to clarify differences between adolescent and adult ethanol exposure and reinforce the need for differential therapeutic approaches.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee on Animal Use—Institute of Biomedical Sciences (#81/2013) of the Universidade de São Paulo.

AUTHOR CONTRIBUTIONS

PC-N and RC designed the experiments, analyzed the data and wrote the manuscript. PC-N and LH conducted the behavioral experiments. JF and CP conducted the measurement and analysis of the dopamine levels by microdialysis.

FUNDING

Funding for this study was provided by grants #2018/05038-0 #2015/02397-0 and 2012/10260-7, São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP). PC-N and LH were the recipient of a scholarship from FAPESP (#2012/17228-1; #2017/11987-1). RC and CP are Research Fellows of CNPq.

ACKNOWLEDGMENTS

We appreciate the dedicated technical work of Elisabete Zocal Paro Lepera from the Universidade Estadual Júlio de Mesquita Filho in São Paulo State, Brazil.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00031/full#supplementary-material.

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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.

frontiers in Behavioral Neuroscience

REVIEW
published: 10 March 2020
doi: 10.3389/fnbeh.2020.00033

Fetal Alcohol Programming of Subsequent Alcohol Affinity: A Review Based on Preclinical, Clinical and Epidemiological Studies

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The anatomo-physiological disruptions inherent to different categories of the Fetal Alcohol Spectrum Disorder do not encompass all the negative consequences derived from intrauterine ethanol (EtOH) exposure. Preclinical, clinical and epidemiological studies show that prenatal EtOH exposure also results in early programming of alcohol affinity. This affinity has been addressed through the examination of how EtOH prenatally exposed organisms recognize and prefer the drug's chemosensory cues and their predisposition to exhibit heightened voluntary EtOH intake during infancy and adolescence. In altricial species these processes are determined by the interaction of at least three factors during stages equivalent to the 2nd and 3rd human gestational trimester: (i) fetal processing of the drug's olfactory and gustatory attributes present in the prenatal milieu; (ii) EtOH's recruitment of central reinforcing effects that also imply progressive sensitization to the drug's motivational properties; and (iii) an associative learning process involving the prior two factors. This Pavlovian learning phenomenon is dependent upon the recruitment of the opioid system and studies also indicate a significant role of EtOH's principal metabolite (acetaldehyde, ACD) which is rapidly generated in the brain *via* the catalase system. The central and rapid accumulation of this metabolite represents a major factor involved in the process of fetal alcohol programming. According to recent investigations, it appears that ACD exerts early positive reinforcing consequences and antianxiety effects (negative reinforcement). Finally, this review also acknowledges human clinical and epidemiological studies indicating that moderate and binge-like drinking episodes during gestation result in neonatal recognition of EtOH's chemosensory properties coupled with a preference towards these cues. As a whole, the studies under discussion emphasize the notion that even subteratogenic EtOH exposure during fetal life seizes early functional sensory and learning capabilities that pathologically shape subsequent physiological and behavioral reactivity towards the drug.

Keywords: fetal alcohol programming, odor and taste, reinforcement, anxiety, associative learning, acetaldehyde, opioid system

OPEN ACCESS

Edited by:
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Reviewed by:
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University of Cagliari, Italy

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Specialty section:
This article was submitted to Learning and Memory, a section of the journal Frontiers in Behavioral Neuroscience

Received: *28 November 2019*
Accepted: *17 February 2020*
Published: *10 March 2020*

Citation:
Miranda-Morales RS, D'Aloisio G, Anunziata F, Abate P and Molina JC (2020) Fetal Alcohol Programming of Subsequent Alcohol Affinity: A Review Based on Preclinical, Clinical and Epidemiological Studies. Front. Behav. Neurosci. 14:33. doi: 10.3389/fnbeh.2020.00033

INTRODUCTION

Two drug-related phenomena act as the foundation for the present review: (i) among other drugs of abuse, ethanol (EtOH) recruits non-associative and associative learning capabilities of the organism; and (ii) age of onset of EtOH-related experiences represents a critical factor determining later use and abuse of this psychotropic agent. Both issues are intrinsically related to the concept of early-life adaptations to environmental disturbances that increase susceptibility to diseases in later life (Barker et al., 2009; Hay, 2009; Calkins and Devaskar, 2011). Under the framework of this vision, we will primarily examine fetal alcohol programming processes, which according to preclinical, clinical and epidemiological studies, represent a critical factor in the structure of subsequent affinity for the drug and the future well-being of the organism. To accrue this goal the present review requires the analyses of the recruitment of early sensory capabilities when the drug invades the prenatal milieu, early EtOH's motivational properties and the likelihood of learning processes combining both factors. Other critical elements that are needed to understand short- and long-lasting consequences of fetal alcohol programming are related with the critical participation of EtOH's principal metabolite (acetaldehyde, ACD), how the endogenous system modulates the reinforcing effects of the drug and the effects of the interaction between prenatal and postnatal experiences that shape alcohol recognition, discrimination and preference patterns.

From a general perspective, there is no doubt that EtOH's teratogenic potential represents the main issue linking embryonic and fetal life with maternal consumption of the drug. The identification of a syndrome (Fetal Alcohol Syndrome, FAS) representing one of the major congenital causes of mental disabilities (Lemoine et al., 1968; Jones et al., 1973) as well as the subsequent recognition and definition of Fetal Alcohol Spectrum Disorders (Riley and McGee, 2005; Riley et al., 2011; Mattson et al., 2019) have guided most of the literature concerning early development and EtOH intoxication. According to the US National Institutes of Health (ncbi.nlm.nih.gov; October 2019), approximately 17,000 articles arise when linking the terms "fetal" and "alcohol." Within this considerable number of peer-reviewed studies, it is possible to detect an early publication that explicitly examined the consequences of fetal chronic exposure to EtOH upon subsequent intake patterns of the drug (Bond and Di Giusto, 1976). According to these authors, and based on an animal model frequently employed to assess EtOH's teratogenic properties, voluntary intake of the drug in an altricial species such as the rat, increases as a function of prenatal exposure to this psychotropic agent. There were methodological problems in this study since experimental pregnant rats consumed a liquid diet containing Sustagen (a milk-based supplement) and ethanol while control mothers were fed on lab chow. This obviously implies possible differential nutritional effects that can also lead to ethanol intake changes in the progeny limiting the conclusions of this study. In addition, given the lack of prior information concerning this phenomenon, it was certainly difficult to determine is such an effect was modulated by factors such as alterations in neurotransmitter systems that sensitize the organism to EtOH's motivational properties (e.g., positive reinforcing effects and/or negative reinforcing effects primarily related with the drug's antianxiety consequences), comorbidity of neurobehavioral disorders (hyperactivity, attentional deficits, depression, impulsivity), disruptions in basic sensory capabilities that impede or alter olfactory and gustatory discrimination processes, etc. Probably none of these modulators can now be dismissed. It is our expectation that the present review will provide at least partially, notions related with the fact that early experiences with the drug recruit functional capabilities of the organism that result in subsequent alcohol affinity. As will be analyzed this recruitment can be established when employing even subteratogenic levels of EtOH exposure. Through the examination of this phenomenon we hope to provide an expansion of the pre-existing vision of neurobehavioral disorders that need to be prevented, diagnosed or treated in children and adolescents with an early positive EtOH history.

The present review will be organized as follows: (i) a first section, primarily centered on preclinical studies, analyses different experimental strategies alluding to fetal capabilities of altricial mammals related to the detection and discrimination of EtOH's sensory attributes; (ii) a second item, also based in animal studies, incorporates the notion of EtOH's reinforcing effects that promote associative learning memories leading to heightened EtOH affinity; (iii) the third section emphasizes the need to consider the drug's principal metabolite (ACD) as a critical factor modulating EtOH's reinforcing effects; (iv) the role of the endogenous opiate system modulating prenatal EtOH reinforcement is additionally considered *via* animal behavioral and psychopharmacological studies; and (v) Finally, the last section of the review is devoted to highlighting human clinical and epidemiological studies endorsing the notion that prenatal EtOH experiences have a major impact on the development of subsequent EtOH affinity. As will be observed, this human section has been organized to depict the analogies and homologies existing between animal and human findings.

DO FETUSES PERCEIVE EtOH'S CHEMOSENSORY PROPERTIES?

Fetal and neonatal sensory discrimination capabilities have been described in different altricial species (rabbits, sheep, rats) including humans (Lecanuet et al., 1995; Schaal et al., 2002, 2004; Clark-Gambelunghe and Clark, 2015; Fulgione et al., 2017). Neuroethological studies indicate that chemosensory systems rapidly become functional *in the uterus* (Molina et al., 1999, 2007a; Schaal et al., 2004; Bloomfield et al., 2017). This development is required for essential survival purposes related to subsequent maternal attachment processes including the discrimination and recognition of the main nutrients (colostrum and milk) that will be provided both peri- and neonatally (Cernoch and Porter, 1985; Makin and Porter, 1989; Marlier et al., 1998; Miller and Spear, 2009; Díaz-Marte et al., 2010; Corona and Lévy, 2015). In accordance with Nicolaidis (Nicolaïdis, 2008) intrauterine experience with flavors derived

from the mother's diet is swallowed by the fetus generating the activity of a functional olfactogustatory system. First experiences with flavors occur prenatally *via* the accumulation of sensory cues in the amniotic fluid yielding olfactory, gustatory and trigeminal stimulation that is also observed when these cues are present in breastmilk (Mennella and Beauchamp, 1991; Beauchamp and Mennella, 2011; Forestell and Mennella, 2017); phenomena that serve to establish orosensory learning processes that reunite the basic characteristics of a prenatal and/or perinatal imprinting process.

Maternal EtOH consumption also results in the accumulation of the drug in the amniotic fluid as well as in breastmilk (Bachmanov et al., 2003; Molina et al., 2007b). The low molecular weight of this psychotropic agent permits its passage through the placenta and the levels attained in the amniotic fluid and fetal blood are comparable to those existing in maternal plasma (Szeto, 1989; Hayashi et al., 1991; Domínguez et al., 1998). As indicated by Glendinning et al. (2017), fetal perception of the drug's chemosensory properties in the rat can be established through two non-mutually exclusive pharmacokinetic mechanisms; humoral and intraoral. The presence of the drug in the bloodstream of the immature organism is capable of generating hematogenic stimulation of chemosensory receptors (Molina and Chotro, 1989a,b; Molina et al., 1989) while the presence of EtOH in the amniotic fluid directly stimulates olfactory, gustatory and trigeminal receptors (Glendinning et al., 2017).

At least four reviews have acknowledged the consequences of fetal exposure to the drug's chemosensory attributes upon later recognition and preference to these cues (Bachmanov et al., 2003; Spear and Molina, 2005; Molina et al., 2007b; Abate et al., 2008). Hence, the present section will only be devoted to summarizing the main findings discussed in such reviews which reinforce the notion that fetal alcohol perception critically intervenes in how the organism later relates to the drug. The following issues will be presented as a function of different experimental strategies analyzing this specific phenomenon.

A first preclinical approach emerges from studies performed in near term rat fetuses (21 gestational days) *via* direct administration of minimal amounts of EtOH in the amniotic fluid (40 μl of a 6% v/v EtOH solution) or a novel scent (lemon). Ten minutes later pups were delivered *via* cesarean surgical procedures (Chotro and Molina, 1990). During the 2nd week of postnatal life, pups were tested in an olfactory preference test as well as in terms of EtOH intake patterns. The sensory cue presented in the amniotic sac was the one preferred in the olfactory evaluation. In addition, pups with a short antenatal EtOH experience were the ones exhibiting heightened levels of EtOH ingestion (Dominguez et al., 1993). Utilizing a similar experimental approach, it was also observed, almost immediately after birth, that pups exposed prenatally to EtOH exhibited clear behavioral and physiological (bradycardia) responses indicative of an orienting response to the smell of the drug (Chotro and Molina, 1992). This antenatal experience, that was not sufficient to generate pharmacologically relevant EtOH levels in the fetus, was also observed to potentiate infantile alcohol-odor conditioned preferences when the odor of the drug was paired with a sweet positive reinforcer (Chotro et al., 1991).

A second experimental approach validating the importance of prenatal exposure to EtOH's sensory properties has been employed during a fetal stage of brain development in the rat analogous to the 2nd human trimester (Dobbing and Sands, 1979; Tran et al., 2000). During this stage rats and humans exhibit a functional olfactogustatory system (Molina et al., 1999; Bloomfield et al., 2017) as well as significant innervations of target fields of the oral trigeminal system (Mbiene and Mistretta, 1997). The procedure normally involves maternal EtOH intoxication utilizing subteratogenic doses (0.5–2.0 g/kg) that are intragastrically administered during the last 4 days of gestation (gestational days 17–20). This protocol yields comparable EtOH levels in the amniotic fluid, fetal and maternal blood (range according to the doses previously mentioned: 50–160 mg/dl; (Domínguez et al., 1996). The first study utilizing this approach indicated that EtOH was not sufficient to cause teratogenic disruptions related to placenta weights, umbilical cord lengths, offspring's body weights, weights and/or size of the olfactory bulbs, cerebral hemispheres, and cerebellum. Furthermore, subsequent research indicates that this EtOH exposure procedure does not affect neural migration processes which allow the appropriate synaptic organization of the glomeruli of the principal olfactory bulbs (Pueta et al., 2011). Yet, fetal early experience with EtOH had a dramatic effect upon perinatal responsiveness to the smell of the drug. Pups pretreated with 1.0 or 2.0 g/kg EtOH consistently showed a marked decrement in their motor activity when stimulated with the scent of the drug. This reaction was markedly different from the one caused by a novel citric scent (Domínguez et al., 1996). We later observed that perinatal infusion of EtOH concentrations similar to those encountered in the amniotic fluid also exerted a sedative effect upon a motor activity (Domínguez et al., 1998). This effect was very similar to the one generated by the amniotic fluid in rat pups with no prior history with the drug. The biological fluid had no sedative effects in pups pretreated with EtOH. In other words, fetal alcohol experience determined a similar profile of perinatal responsiveness to EtOH's sensory cues as the one encountered when control perinates were confronted with a relevant biological sensory cue. Experience with the sensory cues of the amniotic fluid is known to intervene in the acceptance of colostrum and milk (Marlier et al., 1998; Al Aïn et al., 2013; Schaal et al., 2013) or even to act as an appetitive conditioned stimulus (CS; Chotro et al., 2007). These results seem to strengthen the hypothesis of a sensory-imprinting phenomenon generated *via* prenatal EtOH exposure (Nicolaïdis, 2008).

Is this particular prenatal experience sufficient to modify EtOH affinity later in life? The answer is affirmative and once again implies fetal memories relative to EtOH's chemosensory attributes. During infancy, near term rat fetuses exposed to maternal EtOH intoxication not only ingest higher amounts of EtOH but also show drinking preferences when confronted with the configuration of a sucrose-quinine solution (Domínguez et al., 1998). In genetically heterogeneous rats, this gustatory combination has been demonstrated to act as a psychophysical

equivalent relative to the EtOH's taste (Di Lorenzo et al., 1986; Kiefer, 1995; Bachmanov et al., 2003). It has also been demonstrated that heightened EtOH intake patterns are mainly generated when fetal experience occurs during the last two gestational days (19 and 20). This brief temporal exposure is also capable of increasing EtOH palatability (Díaz-Cenzano and Chotro, 2010).

Rat and human neonates detect even minimal amounts of EtOH in maternal milk [rats: 175 mg/dl following maternal intoxication with 2.5 g/kg EtOH (Pepino et al., 1999); humans: 50 mg/dl following the intake of a single beer (Mennella and Beauchamp, 1991, 1993; Mennella, 1997)]. It has also been demonstrated that in rats, EtOH maternal intoxication (1.0 or 2.0 g/kg) during late pregnancy exacerbates offspring's consumption of milk contaminated with the above mentioned minimal amounts of the drug without affecting the consumption of the uncontaminated nutrient (Pueta et al., 2008).

The early recognition of EtOH's sensory attributes as a function of maternal EtOH intoxication during late pregnancy has also been investigated relative to the effects of the drug upon breathing plasticity. Cullere et al. (2015) have observed that brief fetal EtOH experiences in rodents disrupt neonatal breathing patterns when the organism is re-exposed to the toxic effects of the drug. Most importantly, this detrimental effect is exacerbated under the presence of EtOH odor. Similar results have been reported in rats during postnatal days 3–9; a developmental stage that partially overlaps with the 3rd human gestational trimester characterized by a brain growth spurt (Macchione et al., 2016). In subsequent items of this review, these issues will be re-examined as a function of associative learning processes based on EtOH's sensory cues and the drug's motivational effects.

The analysis of sensory-related memories emerging from prenatal EtOH experiences has also been addressed through animal models based on the use of moderate to high levels of EtOH throughout most of the gestational period. The most frequent model that has been utilized is known to exert teratogenic effects and implies a progressive increase in the amount of EtOH provided to the mother *via* a liquid diet (Miller, 1992). Based on this animal model, neurophysiological responsiveness to different odorants including EtOH was mapped across the olfactory epithelium. The main findings observed in infants prenatally exposed to the drug indicate a tuned neurophysiological response to EtOH odor despite an altered general response to alternative odorants. This result was also supported by a specific odorant-induced reflexive sniffing response to EtOH odor emerging from a positive prenatal history with the drug (Youngentob et al., 2007; Eade et al., 2010). Chronic prenatal EtOH exposure also resulted in heightened voluntary EtOH intake during early postnatal life and adulthood (Youngentob et al., 2007). When focusing on the gustatory system, fetal EtOH exposure resulted in the taste-mediated acceptability of EtOH and a diminished aversion to the drug's quinine-like taste component (Youngentob and Glendinning, 2009). In a recent study, the research group of Glendinning et al. (2017) has provided new evidence concerning fetal alcohol exposure reprogramming of the peripheral taste and trigeminal systems. The main findings indicate that prenatal EtOH diminishes aversive flavor attributes of the drug such as its bitter taste and the burning sensation mediated by the trigeminal system.

Sensory persistence of fetal-alcohol related chronic exposure was also assessed during adolescence utilizing an animal social transmission paradigm involving the perception of EtOH odor. The paradigm is based on the interactions between a sober organism (observer) and an intoxicated counterpart (demonstrator) which provides sensory information of the drug *via* direct elimination processes (e.g., alveolar excretion, urination, salivation, et cetera (Fernández-Vidal and Molina, 2004; March et al., 2013d). A positive prenatal alcohol history promoted social interactions with an intoxicated peer and the juvenile re-exposure experience subsequently enhanced EtOH odor response as assessed in a whole-body plethysmograph (Eade and Youngentob, 2009). When configuring the neurophysiological and behavioral studies conducted by Youngentob and Glendinning (2009) the title of one of their articles clearly summarizes the impact of chronic fetal alcohol exposure upon EtOH affinity: "Fetal EtOH exposure increases EtOH intake by making it smell and taste better."

A HOLISTIC VIEW OF FETAL ALCOHOL PROGRAMMING PROCESSES: IS SENSORY FAMILIARIZATION SOLELY RESPONSIBLE FOR SUBSEQUENT EtOH AFFINITY? THE NEED TO CONSIDER EARLY EtOH'S MOTIVATIONAL EFFECTS

According to the information presented in the preceding item, fetuses detect EtOH's sensory attributes and memories arising from these early experiences allow latter discrimination of these cues and preference to such stimuli. Is sensory familiarization solely responsible for subsequent EtOH affinity? From a pharmacokinetic perspective, the studies previously reported based on maternal EtOH intoxication imply the juxtaposition of the drug in the amniotic fluid and its presence in fetal blood and brain. Hence, in terms of potential associative learning processes (e.g., pavlovian or classical conditioning), the requirement of temporal contiguity between specific sensory cues and physiological effects of the drug is met.

It can be argued that when animal studies were conducted *via* direct EtOH contamination of the amniotic fluid while avoiding fetal intoxication (Chotro and Molina, 1990, 1992; Chotro et al., 1991) the main phenomenon determining subsequent EtOH recognition and preference obeyed to familiarization with EtOH's sensory cues. Even under these circumstances, biologically relevant stimuli were found to modulate the memories arising from such experiences. The administration of the drug into the amniotic sac took place only 10 min prior to cesarean delivery. Immediately after delivery, neonatal bodily stimulation was provided to ensure optimal survival rates. This tactile manipulation mimics maternal stimulating effects which, modulated through the endogenous opiate system, acts as an appetitive unconditioned stimulus (US) that is rapidly associated

with pre-existing or simultaneously presented olfactory cues (Leon, 1987; Ronca and Alberts, 1994; Roth and Sullivan, 2006; Raineki et al., 2010). Indeed, when considering EtOH administration into the amniotic fluid and its contingency with activating tactile stimulation, it was observed that sensory preferences were highly dependent upon optimal temporal contiguity between these factors (Molina et al., 1999). In other words, the phenomenon under consideration complied with the notion of the acquisition and retention of a conditioned response.

There are numerous examples that endorse the capability of the fetus to learn conditioned responses when pairing a relatively neutral stimulus (e.g., an odorant) with different biologically relevant events. Conditioned taste aversions have been reported in near term rat fetuses when pairing a tastant (apple juice) with the emetic effects of lithium chloride. These aversions are manifested in multiple ways: avoidance of maternal nipples scented with such tastant or spending less time over shavings that contain this gustatory cue (Stickrod et al., 1982) as well as a noticeable delay to traverse a runaway scented with apple to gain access to the mother (Smotherman, 1982). Acute hypoxia induced by clamping the umbilical cord can also lead to odor conditioned aversions (Hepper, 1991) while on the contrary, cessation of hypoxia leads to odor conditioned preferences (Hepper, 1993).

EtOH can be considered as a drug that depending on factors such as dose, age, genetic predisposition, comorbidity with other neurobehavioral disorders, etc. can exert differential triphasic motivational effects. EtOH intoxication promotes positive and antianxiety effects as well as aversive consequences (Pautassi et al., 2009). Positive reinforcing effects play a significant role in the initiation, maintenance of alcohol intake, seeking behavior of the drug as well as in patterns of abuse of this psychotropic agent. These processes can also be modulated by that the drug's amelioration of aversive states such as anxiety and dysphoria. The progressive maturation of the hepatic system allows the accumulation of peripheral ACD which exerts both gastrointestinal distress and sedative effects known to act as aversive stimuli (Cunningham et al., 2000). Each of these motivational effects can support associative learning given its temporal association with interoceptive or environmental CSs.

When the pregnant female is intoxicated, it is difficult to discern whether fetuses only learn about the sensory components of the drug, its unconditioned effects or the association between these factors. The first animal study that was meant to address these possibilities was originally based on a pharmacokinetic approach involving the distribution of a non-EtOH olfactory cue (cineol, the main component of eucalyptus oil) that easily crosses the placenta and exerts no physiological consequences on the fetus. Cineol can be easily traced in terms of concentration and temporal duration in the amniotic fluid. The strategy was to explicitly associate or not the presence of this CS with the state of fetal EtOH intoxication. These experiences took place during gestational days of 17–20. Three weeks later pups originally exposed to the explicit association between cineol and EtOH intoxication exhibited conditioned orofacial responses when re-exposed to the odorant (Abate et al., 2000). Utilizing a similar strategy, a test was conducted based on the newborn's first suckling response. The test consisted in the evaluation of neonatal attachment to a surrogate nipple that delivers milk. In pups prenatally exposed to an optimal contingency involving cineol and EtOH intoxication, the presence of the odorant promoted heightened suckling behavior of the surrogate nipple. In addition, this effect was potentiated if neonates were re-exposed to a similar state of intoxication as the one experienced *in utero*; a phenomenon that appears to indicate reactivation of the original associative memory (Abate et al., 2002).

The hypothesis of associative learning mechanisms received additional support through a series of preclinical studies where cesarean delivered pups were tested utilizing two alternative conditioning procedures. The first one utilized the surrogate nipple as a CS paired with an intraoral infusion of EtOH while the second one employed an olfactory CS associated with a similar infusion. Pups exhibited pharmacologically relevant levels of EtOH in blood and in both cases, the presence of the odorant elicited heightened responsiveness to the surrogate nipple (Cheslock et al., 2001). The efficacy of EtOH as a positive reinforcer was also detected when utilizing either intraperitoneal or intracisternal (directly into the cisterna magna) administrations of the drug (Petrov et al., 2003; Nizhnikov et al., 2007).

Taking into account the preceding studies, new experimental approaches were focused on the interaction between prenatal experience with the drug and its subsequent reinforcing effects. Near term rat fetuses were exposed to a moderate EtOH dose and following cesarean delivery they were subjected to paired or unpaired experiences comprising a surrogate nipple and different EtOH doses (0.00, 0.25, 0.50 or 0.75 g/kg) which were intraperitoneally injected. A positive antenatal history with the drug increased the magnitude of appetitive conditioned responses to the nipple mediated by EtOH intoxication and also increased the range of doses yielding a reinforcing effect (Nizhnikov et al., 2006). This phenomenon alludes to a sensitization effect relative to the drug's motivational properties.

Further evidence indicative of prenatal EtOH sensitization was detected in infants subjected to an operant conditioning procedure where a sweet tastant (sucrose) served as a reinforcer. After operant training, a moderate EtOH dose (0.5 g/kg) was paired with the sweet taste. In pups prenatally exposed to EtOH, this association enhanced sucrose reinforcement and also increased resistance to extinction of the operant response (Culleré et al., 2014). Progressive sensitization to the US facilitates associative learning processes probably by impeding habituation to conditioned signals and strengthening the contingency between these signals and the US (Çevik, 2014). In the case of early EtOH exposure, this entanglement of non-associative and associative learning effects may help to explain the persistence of memories leading to EtOH affinity. Heightened voluntary adolescent EtOH consumption has been detected following brief experiences with the drug during late gestation (Fabio et al., 2015) as well as when drug exposure occurred during most of the gestational period (Eade et al., 2016). In the first case, it was also observed that prenatal EtOH exposure also mitigated the aversive effects of relatively high

doses of the drug that are sufficient to generate conditioned taste aversions. Furthermore, late prenatal EtOH exposure has been observed to decrease neural activity within the infralimbic cortex, an area implicated in the extinction of drug-mediated associative memories (Fabio et al., 2013).

Heightened early sensitivity to EtOH's reinforcing effects has also received support from operant conditioning procedures adapted to the sensory and motor capabilities of the perinate. It was originally demonstrated that perinatal behaviors (nose-poking and front limb movements) deployed to stimulate the mammary gland and to facilitate nipple attachment increase as a function of intraoral delivery of maternal milk (Arias et al., 2007). This technique was adapted to assess the reinforcing value of different EtOH solutions. Operant conditioning mediated by the drug rapidly occurred and perinates exhibited dose-dependent blood EtOH concentrations. At the beginning of an extinction process, pups reinforced with EtOH were also found to exhibit exacerbated seeking behaviors of the drug (Bordner et al., 2008). As assessed through this operant paradigm, late prenatal exposure to EtOH enhances the reinforcing effects of the drug as well as of a taste-related psychophysical equivalent such as the configuration between sucrose and quinine (March et al., 2009). This taste configuration has no reinforcing effects in pups without prior prenatal EtOH exposure. Apparently, the association between EtOH's sensory attributes and the reinforcing effects of the drug allows the relatively neutral gustatory components later act as second-order reinforcers (Molina et al., 2007a).

Notice that the above-summarized studies seem to indicate that EtOH primarily acts as the appetitive US when employing classical conditioning paradigms or as a positive reinforcer when examining animal operant learning. In some of these studies, EtOH doses yielding relatively high blood and brain levels of the drug were employed. In no case, conditioned taste or odor aversions were detected. These aversions have been observed during infancy, adolescence and adulthood. More specifically, EtOH-mediated conditioned aversions are rapidly established in 10-day-old or older rats (for a detailed ontogenetic revision on this matter see Pautassi et al., 2009). Some studies have utilized high EtOH doses (e.g., 3.0 g/kg) during stages equivalent to the 2nd or 3rd human gestational trimester. In older animals, these doses exert highly aversive effects that seem to provoke emetic-like effects as the ones induced by lithium chloride (Arias et al., 2007; Pautassi et al., 2008). One of these studies was conducted during late prenatal life where pregnant females received binge-like exposures to the drug operationalized through intragastric administration of a 3.0 g/kg dose (peak blood and amniotic fluid levels were approximately equivalent to 200 mg/dl; Chotro et al., 2009). Direct elimination of the drug (e.g., alveolar excretion and salivation) or hematogenic stimulation of sensory receptors promotes associative learning comprising EtOH's chemosensory cues and the drug's interoceptive effects (Molina et al., 1989). As described, fetuses exhibit associative learning given the contingency between sensory and toxic effects of EtOH. Dams treated with the above-mentioned dose later exhibited an initial rejection of an EtOH solution in a voluntary intake test suggestive of a conditioned orosensory aversion. On the contrary, their offspring showed alcohol affinity during infancy.

As can be observed, noticeable ontogenetic differences emerge when considering the motivational properties of the drug. Fetuses as well as neonates (7–8 day old rats) treated with a high EtOH dose (3.0 g/kg) later exhibit enhanced drug palatability and heightened predisposition to consume this psychotropic agent. At 10 days of age opposite outcomes (disgust reactions and intake decrements) are detected following toxic experiences generated by a similar dose (Arias and Chotro, 2006). As indicated by the authors of this last study, there exists an ontogenetic change in the perception of EtOH's reinforcing properties that coincides with the end of a sensitive period for learning preferences in pups younger than 9 days of age (Sullivan et al., 2000; Roth and Sullivan, 2003). During this sensitive period, certain neurobiological factors (immaturity of the amygdala, low brain corticosterone levels) weaken the capability of the organism to learn certain aversions (Debiec and Sullivan, 2017). Nevertheless, when considering EtOH intoxication there are other important metabolic factors that should be considered when examining the early predisposition to learn conditioned preferences and to exhibit resistance to the drug's aversive effects. This topic focuses on the motivational role of EtOH's first metabolite (ACD) during early ontogeny.

ACETALDEHYDE METABOLISM DURING EARLY ONTOGENY: EARLY MOTIVATIONAL EFFECTS OF EtOH'S PRINCIPAL METABOLITE

Peripheral and central production of ACD critically modulates sensitivity to EtOH's motivational properties. In genetically selected animals in terms of alcohol affinity as well as in genetically heterogenous rats and mice, the central production of ACD *via* the catalase system represents a critical factor modulating EtOH's positive reinforcing effects (Quertemont, 2004; Israel et al., 2015; Peana et al., 2015). The induction of the activity of this enzymatic system potentiates EtOH's motor stimulating effects (Correa et al., 2001). This stimulatory effect has been considered as an index of positive rewarding central effects of different drugs of abuse (Wise and Bozarth, 1987; Orsini et al., 2004). Correa et al. (2001) showed that in adult rodents pre-treatment with 3-amino-1H,2,4-triazole, a well-known catalase inhibitor, before EtOH administration, significantly reduces EtOH-induced motor stimulation as well as brain catalase activity. In the periphery, EtOH is mainly metabolized into ACD *via* hepatic alcohol dehydrogenase enzymes and the accumulation of the metabolite has been primarily associated with aversive motivational effects (Escarabajal et al., 2003; Sanchis-Segura et al., 2005). Given the hepatic immaturity of fetuses and neonates, the capability to metabolize EtOH into ACD is markedly lower than the one observed during infancy, adolescence and adulthood (Kelly et al., 1987). On the contrary, the activity of the central catalase system negatively correlates with age. The peak activity

level of this brain enzymatic system is observed during late gestation and in neonates (Del Maestro and McDonald, 1987). Given this pharmacokinetic profile during early ontogeny, EtOH intoxication implies: (i) a significant accumulation of ACD in the brain; a factor positively correlated with EtOH's reinforcing effects; and (ii) hepatic immaturity delaying peripheral accumulation of the metabolite which potentially reduces the aversive effects of the drug upon the gastrointestinal system (March et al., 2013b). Considering both processes it appears that, from a metabolic perspective, the balance between central and peripheral ACD production and accumulation during early ontogeny favors positive motivational effects of EtOH.

Different studies based on early EtOH or ACD central administration validate the notion that the metabolite is critical in terms of determining or modulating positive reinforcement. In cesarean delivered rat neonates, only one conditioning trial where an odor was associated with central EtOH administration, was sufficient to generate an olfactory conditioned preference. This effect was completely absent when the catalase system was inhibited with sodium azide (Nizhnikov et al., 2007). Sodium-azide treatment did not affect motor activity when pups were stimulated with the olfactory CS during conditioning or when evaluating nipple attachment at the test; results indicating that neither perception of the CS or the pup's motor capabilities changed as a function of this drug treatment. Despite these null effects, a follow-up experiment was conducted to test whether the inhibitory effects of sodium azide upon EtOH reinforcement were specific relative to catalase inhibition or if they were merely related to negative consequences upon sensory or learning capabilities of the organism. Rather than employing EtOH as a reinforcer, the study was conducted with the central administration of an endogenous kappa opioid receptor agonist (dynorphin A-13) also known to exert positive reinforcement early in life. The effects of this agonist are independent of metabolic processes corresponding to the catalase system. The study clearly indicated the reinforcing effects of dynorphin that were not affected by inhibition of the catalase system.

Based on these results, studies were performed in cesarean delivered pups intracisternally administered with an EtOH dose (100 mg%) that exerts positive reinforcing effects or an ACD dose (0.35 μ mol) also known to exert psychomotor stimulating effects in adults. Both EtOH and ACD activate the mesolimbic dopamine system which is critical in mediating reinforcing effects of different drugs of abuse (Melis et al., 2007; Diana et al., 2008). Brain administration of the drug or its first metabolite was sufficient to promote the rapid acquisition of conditioned olfactory preferences which was markedly inhibited when sequestering central ACD through the use of d-penicillamine (March et al., 2013a,c). A recent study also showed that maternal administration of d-penicillamine significantly decreases the prenatal reinforcing effects of EtOH when considering different levels of expression of alcohol affinity: (i) attractiveness to EtOH odor as assessed through an odor crawling locomotion neonatal test; (ii) operant responding supported by EtOH reinforcement at postnatal day 5; and (iii) EtOH intake during the second postnatal week (Gaztañaga et al., 2017).

Further support relative to the importance of ACD in the regulation of EtOH's reinforcing effects was provided by a meta-analytical perspective. The question that guided this approach was the examination of a possible correlation existing between levels of catalase activity and EtOH affinity across ontogeny. As a first step, developmental changes in catalase activity based on average scores (U/mg protein) observed in cerebral hemispheres, striatum, cerebellum and brain stem were taken into account (Del Maestro and McDonald, 1987). As previously mentioned, there is a gradual decrease in the levels of catalase activity as a function of increasing age. Values corresponding to different age groups were linearly correlated with blood EtOH levels derived from alcohol consumption tests performed at similar ages (Truxell and Spear, 2004; Truxell et al., 2007). Spontaneous EtOH intake also decreases gradually across development. This inferential strategy indicated highly significant positive correlations when considering voluntary consumption of 15% v/v (Pearson's correlation coefficient, $r = 0.82$) or 30% v/v EtOH solutions ($r = 0.93$; March et al., 2013b).

Beyond EtOH's positive reinforcing effects, is it possible that the antianxiety properties of the drug also participate in the structure of early EtOH-related memories? Antianxiety effects of EtOH have been observed in infant rats. Ultrasonic vocalizations caused by the stress of isolation or derived from a given forced administration procedure (e.g., intraperitoneal, intracisternal or intragastrical) significantly decrease when delivering low to moderate EtOH doses (Pautassi et al., 2007, 2012). Infantile aversive conditioned responses have also been observed to decrease, whenever EtOH is paired with the aversive US; a learning process known as devaluation (Pautassi et al., 2006). Both phenomena argue in favor of early sensitivity to the drug's antianxiety effects which are similar to those observed when using a GABA-A receptor agonist such as midazolam (Pautassi et al., 2007). Despite these considerations, the involvement of EtOH's antianxiety properties has not been directly assessed during prenatal or early postnatal life.

Recent studies performed during the stage equivalent to the 3rd human gestational trimester have suggested the implication of EtOH's antianxiety effects in associative learning processes. The studies have been conducted during postnatal days 3, 5, and 7 in the rat and they were principally aimed at analyzing EtOH's effects upon neurorespiratory plasticity. During this and earlier stages in development, the immature organism learns an association between different ambient cues and the drug's depressant effects upon respiration (Cullere et al., 2015; Macchione et al., 2016). We also observed that stressors like maternal deprivation, exposure to a novel context and intragastric or intracisternal administrations had a major disruptive impact upon breathing patterns (Acevedo et al., 2017; Macchione et al., 2018). Recently, utilizing a tactile discrimination procedure we found that intragastric administration of a vehicle solution in maternally deprived pups generated a significant increase in the level of apneic episodes. These disruptions, probably caused by an abnormal level of arousal, were significantly attenuated when EtOH (2.0 g/kg) rather than the vehicle was administered. In this study,

two groups of pups were defined by either EtOH or vehicle administration paired with a salient texture across days. A third group received EtOH during the first 2 days of training while the third-day vehicle was administered. Pups in this last group, despite receiving a vehicle, showed similar attenuations of the apneic episodes as those always treated with the drug. This effect appears to indicate a conditioned respiratory response probably associated with the preceding experiences with the antianxiety effects of the drug. It is also important to note that those pups that were always treated with vehicle later exhibited very low levels of preference towards the tactile cue associated with the administration procedure. This apparent conditioned aversion was completely absent in pups previously exposed to the drug (D'Aloisio et al., 2019). Given these results, it can be argued that fetal alcohol programming leading to subsequent EtOH affinity implies the interaction between two non-mutually exclusive motivational properties of the drug: it's positive as well as its antianxiety effects.

To our knowledge, the role of ACD in the early modulation of EtOH's anxiolytic effects has not been analyzed. In adult organisms of different altricial species, this phenomenon has been, at most, scarcely analyzed. The results in adults are controversial and dependent upon dosing factors and the site of administration of the metabolite or of drugs that affect the process of EtOH metabolism. Given the marked ontogenetic changes concerning peripheral or central EtOH metabolism, it is also difficult to extrapolate what is known in adults to early ontogenetic stages. Considering this important limitation, the adult literature endorses the anxiogenic effects of the metabolite following its ingestion (Plescia et al., 2015) as well as when the metabolite is intraperitoneally administered (Escrig et al., 2012). When ACD peripheral metabolism was inhibited through cyanamide there were no indications that ACD participates in EtOH-induced anxiolytic effects (Tambour et al., 2005). Focusing on cerebral metabolism of EtOH into ACD, the inhibition of this process or the inactivation of ACD, have a suppressive effect on the anxiolytic actions of EtOH (Correa et al., 2008). As can be observed and considering the limitations of ontogenetic comparisons, there is not enough evidence to understand the role of the metabolite relative to EtOH's anxiolytic properties.

ROLE OF THE OPIOID SYSTEM IN THE MODULATION OF PRENATAL EtOH REINFORCEMENT

EtOH induces the release of opioids (Herz, 1997; Gianoulakis, 2001) and the endogenous opioid system plays a major role in EtOH reinforcement (Méndez and Morales-Mulia, 2008). EtOH reinforcing effects are mediated mainly through the stimulation of the mu (MOR) and delta (DOR) opioid receptors (Acquas et al., 1993; Herz, 1997). In infant and adult nondependent rodents, kappa opioid receptors (KOR) appears to mediate the aversive properties of alcohol (Land et al., 2009) and may contribute to EtOH's anxiolytic effects (Walker and Koob, 2008). The NOP receptor (nociceptin/orphanin FQ peptide) also appears to be critically involved in alcohol affinity. Pharmacological studies indicate that nociceptin and other NOP agonists are effective in reducing EtOH drinking (Martin-Fardon et al., 2010) and reinforcement (Miranda-Morales et al., 2013; Miranda-Morales et al., 2014).

Regarding the effects of prenatal EtOH exposure, general blockade of the opioid system by administration of naloxone to pregnant intoxicated rat dams inhibits the facilitative effect of prenatal EtOH exposure on subsequent EtOH reinforcement in neonates (Miranda-Morales et al., 2010), infants (Chotro and Arias, 2003; Gabriela Chotro and Arias, 2007; Miranda-Morales et al., 2010) and adolescents (Chotro and Arias, 2003). Furthermore, MOR prenatal blockade completely inhibited the facilitative effect on EtOH intake and palatability, while KOR prenatal antagonism only partially reduced the palatability effect (Díaz-Cenzano et al., 2014). Nevertheless, alcohol odor attraction in neonates prenatally exposed to the drug is attenuated by mu or kappa antagonism (Youngentob et al., 2012; Gaztañaga et al., 2015). In summary, this evidence implies activation of the endogenous opioid system following fetal alcohol exposure and the involvement of opioid activity in order for EtOH to function as an effective positive reinforcer during early ontogeny. It is necessary to observe that these effects, as well as the ones that will later be reported, have been encountered when utilizing relatively low to moderate EtOH doses during late gestation. As previously stated, these levels of intoxication fail to exert morphological alterations or to significantly affect the sensory, behavioral or learning capabilities of the developing organism (Molina and Chotro, 1989b; Molina et al., 1989; Lecanuet et al., 1995). In other words, the animal studies under consideration do not meet the criteria of FAS or FASD-like phenotypes as those indicated by Petrelli et al. (2018). Beyond these considerations, it is necessary to observe that animal studies based on chronic and high EtOH doses during gestation have indicated severe alterations in the endorphin system that results in hyperresponsiveness to stressors, a phenomenon that may also lead to heightened EtOH consumption given the antianxiety effects of the drug (Weinberg et al., 1996; Sarkar et al., 2007).

Brief prenatal exposure to moderate EtOH doses significantly reduces opioid peptide concentrations, indicating a possible mechanism by which fetal intoxication can affect future responsiveness towards EtOH (Bordner and Deak, 2015). Other fetal-related studies also indicate selective changes in Methionine-enkephalin (MET-ENK) levels in regions of the mesocorticolimbic and nigrostriatal systems, the hypothalamus and hippocampus showing the involvement of enkephalins in EtOH reinforcement (Abate et al., 2014). MET-ENK levels are specifically changed by fetal intoxication in the above-mentioned central areas during adolescence. These neural enkephalinergic changes modulate later adolescent sensitivity to different motor effects of EtOH (Abate et al., 2017). Interestingly, fetal alcohol experiences also increased levels of mu-opioid receptor transcripts after intake without affecting DOR or KOR receptor transcripts in adolescents (Fabio et al., 2015). MOR mRNA levels revealed differences in infant rats tested in terms of EtOH consumption. This effect was observed when comparing prenatally manipulated subjects (mothers administered with either EtOH or vehicle during late gestation) vs. unmanipulated

animals. Consequently, prenatal manipulation promotes changes in MOR mRNA expression (Guttlein et al., 2019). In this study, MOR mRNA levels increased in infant rats following the consumption of the drug. Moreover, prenatal intragastric manipulation during late pregnancy (that can act as a mild stressor) inhibited the increment of MOR mRNA when pups were tested in an EtOH intake test (Guttlein et al., 2019). These studies suggest a key role of MOR and the enkephalinergic systems in EtOH-mediated reinforcement during early ontogeny. In addition, fetal alcohol experiences modulated these opioid sub-systems in terms of further responsiveness to EtOH.

The kappa opioid system is of special interest when regarding fetal alcohol exposure in part because of an established functional switch during ontogeny, from mediating appetitive during early infancy (Petrov et al., 2006) to aversive motivated behaviors (Shippenberg and Herz, 1986; Bals-Kubik et al., 1989). Fetal intoxication also seems to affect KOR function and expression. Infants prenatally exposed to EtOH exhibited either no aversion or appetitive responding to KOR activation, while control subjects reacted aversively following KOR activation. Furthermore, following antenatal experience with the drug, synaptosomal KOR expression was down-regulated in brain areas implicated in the motivational effects of the drug such as the nucleus accumbens, amygdala, and hippocampus (Nizhnikov et al., 2014). As previously mentioned, prenatal EtOH may trigger transient alterations in KORs during early ontogeny. These alterations mimic the levels of KOR mRNA expression in nucleus accumbens, infralimbic cortex and ventral tegmental area in adolescents prenatally exposed to the drug (Fabio et al., 2015). Bordner and Deak (Bordner and Deak, 2015) reported a similar transient result: a heightened expression of prodynorphin (PDYN, the gene coding for the pre-protein that yields dynorphins, the endogenous ligands of KOR) mRNA transcript in ventral tegmental area in rats prenatally exposed to the drug at postnatal day (PD) 4 but not at PDs 8 or 12.While the underlying mechanisms for these effects are not yet clear, it has been recently reported an increase in mRNA levels of KOR and PDYN in the ventral tegmental area of infant and adolescent rats prenatally exposed to EtOH. Epigenetic modifications seem to explain the changes in gene expression since these effects were associated with a reduction of DNA methylation at PDYN and KOR gene promoters (Wille-Bille et al., 2018). Alternative studies also showed that gene expression from the hypothalamus of rat pups (unmanipulated during gestation) exhibited a down-regulated expression of PDYN mRNA and up-regulated mRNA expression of KOR when the first experience with EtOH was defined by infantile consumption of the drug (Guttlein et al., 2019). These results support the hypothesis of Walker and Koob (2008) that repeated EtOH exposure activates an anti-reward system that sensitizes the organism to stress and anxiety-related stimuli and, in turn, promotes a particular sensitivity to EtOH's anxiolytic effects.

Further research is required to investigate the interconnection between prenatal ACD and the opioid system. D'Addario et al. (2008) reported changes in the expression of the opioid receptors and the precursors of their ligands in response not only to EtOH but also to ACD exposure in human neuroblastoma cells. ACD represents the possible candidate by which EtOH increases the release of β-endorphin which, in turn, can modulate the activity of other neurotransmitter systems such as mesolimbic dopamine (Font et al., 2013). In fact, it has been demonstrated that the opioid system participates in ACD-induced increments in dopaminergic neuronal activity (Fois and Diana, 2016). Specifically, opioid antagonists (naloxone and naltrexone) were found to abolish ACD-induced increase in the firing rate and bursting activity of the dopaminergic neurons of the ventral tegmental area.

The hypothesis that the interaction between ACD and the opioid system in the early regulation of EtOH affinity is also supported, at least indirectly, by preclinical approaches where EtOH maternal intoxication occurred during late prenatal life. As stated, this procedure results in subsequent increases in drug palatability and EtOH intake (Schaal et al., 2013); effects that are eliminated when the state of intoxication is preceded by the administration of the opioid antagonist naltrexone (Miranda-Morales et al., 2014). Interestingly, when utilizing the same animal model but, in this case, sequestering ACD *via* D-penicillamine in fetuses under the state of EtOH intoxication, completely eliminates later affinity for the drug (March et al., 2013c).

HUMAN STUDIES ENDORSING FETAL ALCOHOL PROGRAMMING OF SUBSEQUENT ALCOHOL AFFINITY

When considering human-based literature it is also clear that the fetus processes non-biological volatile substances present in the amniotic fluid. For example, when anise is incorporated in the maternal diet during the last 2 weeks of pregnancy, infants exhibit a stable preference for this odorant while non-exposed controls rather exhibit aversive responding (Schaal et al., 2000). There are also indications that during neonatal life the smell of the amniotic fluid is more effective in guiding nipple attachment even when compared with the natural scent of the mother's breast (Varendi et al., 2010). When considering the process of lactation, the excellent work of Mennella and Beauchamp (1991, 1993) and Mennella (1998) has demonstrated that human babies not only process minimal amounts of alcohol in maternal milk but also that this experience enhances alcohol odor preferences.

The first human study that was conducted relative to EtOH exposure during fetal life and later neonatal alcohol recognition and discrimination was performed in the 2,000 year (Faas et al., 2000). It is important to note that in this study none of the participants exhibited excessive EtOH intake patterns during pregnancy and all the neonates under evaluation (postpartum age: 24–48 h) were considered as completely healthy as a function of the pertinent clinical and biochemical analyses. Mothers were classified as infrequent or moderate drinkers. This last group was constrained to mothers that drank at least once a week and their consumption scores per occasion averaged approximately 22 g of 190 proof alcohol. Newborns were sequentially stimulated with a cotton

swab placed close to the nostrils containing 0.16 g of alcohol or a similar amount of a novel scent (lemon). Head and body movements were video recorded. No differences emerged when contrasting motor activity patterns elicited by lemon odor as a function of maternal alcohol drinking patterns. When employing alcohol odor, babies born to moderate drinkers displayed heightened body, head and facial movements. Subsequently, a new study was conducted using similar experimental procedures and criteria but in this case, the Neonatal Facial Action Coding system was employed (Faas et al., 2015). Facial expressions indicative of appetitive (suckling, smiling and tongue protrusion) or aversive (gaping, brow and nose wrinkling and eye blinking) facial expressions were recorded by blind experimenters. When stimulated with alcohol odor, newborns delivered by moderate drinkers exhibited significantly higher frequencies of appetitive facial responsiveness (particularly when considering tongue protrusion) when compared to babies representative of abstemious or infrequent drinkers. From a correlational perspective the overall amount of alcohol consumed during pregnancy positively and significantly correlated with appetitive reactivity. These results have been recently validated and extended in a clinical investigation where binge drinkers during pregnancy were also included (Anunziata et al., 2019 under revision). Babies born to these mothers were the ones exhibiting the highest levels of appetitive facial expressions, coupled with diminished aversive responding when confronted with alcohol odor. Once again, differential patterns of alcohol use during pregnancy had no effect when newborns were exposed to a novel scent. In summary, moderate or relatively high levels of alcohol intake during pregnancy promote EtOH odor recognition, discrimination and heightened hedonic responses in human newborns.

Does preference for EtOH's sensory attributes as a function of prenatal exposure to the drug persists in subsequent human ontogenetic stages? The first observation that appears pertinent is that 6-16-year-old children or adolescents heavily exposed to alcohol *in utero* (at least four drinks per occasion at least once a week or 14 drinks per week during gestation) exhibit poor performance in the San Diego Odor Identification Test. None of these subjects was diagnosed with FAS and interestingly, the evaluation did not include EtOH odor (Bower et al., 2013). Despite these olfactory deficits derived from EtOH intrauterine exposure, young adults (18–19 years old) born to mothers that drank at least two standards drinks per day during pregnancy, exhibit heightened relative ratings of pleasantness for alcohol odors (Hannigan et al., 2015). More specifically, higher levels of prenatal alcohol exposure were related to higher ratings of pleasantness for the scent of the drug. The importance of this result is highlighted when considering several factors systematically controlled by the authors of the study. Among others, the study is characterized by detailed information relative to maternal drinking patterns based on beverage type, specific drinking habits, binge drinking episodes, number of standard drinks at particular times of the day and days of the week when the drug was consumed. Participants (young adults with differential levels of prenatal EtOH exposure) were assessed in terms of current risk alcohol use while other control variables were also taken into account (e.g.,: home environment, parenting quality, maternal age at the time of the first prenatal visit, IQ, education, and prenatal exposure to other drugs of abuse such as nicotine, marijuana, and cocaine). When considering the olfactory assessment procedures, participants were evaluated using the "Bottle Test" based on studies performed by Schmidt and Beauchamp (1988) and Mennella and Garcia (2000) which allows the evaluation of olfactory identification in conjunction with the possibility of determining levels of pleasantness elicited by a given odor stimulus. The University of Pennsylvania Smell Identification Test (Doty et al., 1984a,b, 1989; Doty and Agrawal, 1989) was also employed to control possible olfactory dysfunctions as anosmia or mild, moderate, or severe smell loss. The main finding related to the significant association existing between prenatal alcohol exposure and alcohol odor preference endorses the hypothesis of prenatally acquired memories that are retained for long periods of time despite a considerable number of interceding postnatal experiences.

From an epidemiological perspective, approximately a decade ago, Foltran et al. (2011) systemically reviewed the literature linking prenatal maternal drinking and alcohol use and abuse during postnatal life. Seven studies were taken into account; two of them were conducted in Australia (Alati et al., 2006, 2008a,b) while the remaining investigations were performed in the USA (Baer et al., 1998, 2003; Griesler and Kandel, 1998; Yates et al., 1998; Barr et al., 2006). All of these studies with the exception of the one conducted by Yates et al. (1998; case-control investigation) were based on prospective cohort analysis. Evaluations relative to EtOH affinity focused on different dependent variables; among others, children's drinking patterns at age 14 and onset of alcohol disorders during adolescence and young adulthood. Different covariates were taken into account (e.g., family and maternal history of psychopathological disorders, prenatal consumption of other drugs of abuse, socioeconomic and educational as well as different developmental parameters at birth and during the childhood of the participants under evaluation). As explicitly mentioned in Foltran's review (Foltran et al., 2011) the overall results of these epidemiological studies "... seem to provide support for a biological origin of some cases of early drinking through a 'programming' effect on the brain's natural reward circuitry. They confirm emerging evidence pointing to *in utero* alcohol exposure, at least at high doses, in the development of addictions."

Recent epidemiological studies have also strengthened the hypothesis stating that intrauterine EtOH exposure leads to later alcohol use and abuse. In a prospective longitudinal study conducted by Goldschmidt et al. (2019), offspring were evaluated from birth to young adulthood as a function of maternal consumption during the 1st, 2nd and/or 3rd trimester of pregnancy. Relative to higher levels of drinking and Alcohol Use Disorders by age 22, the study concludes that prenatal exposure to even one drink per day significantly augments these problematic consequences. In Sweeden, it has also been observed that individuals diagnosed with FAS are more likely to be hospitalized for alcohol abuse disorders (9% vs. 2%

corresponding to healthy control subjects; Rangmar et al., 2015). In a sample of a low-income African-American population in the USA, there have also been indications that prenatal alcohol exposure, especially in young adult males, leads not only to legal difficulties but also to heightened alcohol use. In this study, higher levels of alcohol use were encountered in prenatally exposed subjects that do not exhibit physical or cognitive deficits. When contrasting these results with those reported for example by Rangmar et al. (2015), it appears that alcohol drinking patterns and alcohol use disorders dependent upon gestational exposure to the drug are also modulated by different intervening variables. Ethnicity, alcoholism's genetic predisposition, socioeconomic and educational status, sensitivity to EtOH's disruptive effects upon externalizing traits (e.g., low shyness, hyperactivity, attention deficits and conduct problems in childhood and early adolescence) should not be dismissed when determining alcohol use and abuse disorders in fetuses exposed to the drug (Kendler et al., 2013).

In agreement with Foltran et al. (2011), the human literature is congruent with those preclinical studies previously described relative to the concept of fetal alcohol programming of subsequent alcohol affinity. In prior sections, we have emphasized functional sensory and learning capabilities (sensitization to EtOH's motivational effects and associative learning processes) of the unborn organism that generate specific alcohol-related memories. These processes in no way exclude alternative or complementary mechanisms that may also intervene in the early generation of alcohol programming. One of these mechanisms is related to the dysregulation of the hypothalamic-pituitary-adrenal axis of animals and humans prenatally exposed to the drug (Hellemans et al., 2010) that eventually leads to anxiety, depression, conduct disorders and emotional disorders (Easey et al., 2019). The comorbidity between these disorders and alcohol use and abuse is well known (Kingston et al., 2017; Oliveira et al., 2018).

CONCLUDING REMARKS

The present review focuses on the functional capabilities of the unborn organism that are recruited following alcohol maternal consumption. Both, the animal and human literature consistently demonstrate that fetuses acquire information of EtOH's chemosensory attributes even when minimal amounts of the drug stimulate olfactory or gustatory receptors. Prenatal exposure to moderate or even high EtOH doses has also been observed to sensitize the organism to the drug's motivational properties; particularly its positive reinforcing effects or its anxiolytic effects modulated by the endogenous opiate system. The central accumulation of EtOH or of its principal metabolite (ACD) exert unconditioned motivational effects that are rapidly associated with the drug's sensory cues leading to long-lasting memories that impact upon later alcohol affinity even in terms of generating patterns of drug abuse. As discussed, the process of Fetal Alcohol Programming might occur independently from the teratological effects of the drug.

Fetal Alcohol Programming should broaden our knowledge of potential alterations that are still not taken into account in the diagnostic criteria of FASD. Behavioral signs of Fetal Alcohol Programming have been already detected through relatively simple neonatal techniques developed for altricial mammals including humans (Abate et al., 2008; Faas et al., 2015). These considerations coupled with the ample range of physical, behavioral and cognitive deficits caused by intrauterine EtOH exposure should emphasize the urgent need for more profound and ample primary prevention strategies of the consequences of fetal alcohol exposure. This last statement is even more relevant when considering at least three factors: (i) Internationally, approximately 10% of women consume alcohol while pregnant. One of every 67 of these women delivers a child diagnosed with FAS (Popova et al., 2017); (ii) fertile adolescents and young adults constitute a population segment particularly vulnerable when considering alcohol use and abuse that increases the risk of delivering a child with FASD or programmed in terms of alcohol affinity; and (iii) Re-exposure to the drug during lactation or subsequent stages in development (childhood or adolescence) also constitutes a risk factor in terms of potentiating the imprinting-like phenomenon here referred as Fetal Alcohol Programming [lactation: (Pepino and Mennella, 2004); (Pueta et al., 2008); infants: (Culleré et al., 2014); adolescents: (Eade and Youngentob, 2010)].

AUTHOR CONTRIBUTIONS

JM, RM-M, and PA conceptualized and designed the revision and drafted the sequential versions of the manuscript. GD'A and FA participated in the writing process of the revision as well as in the appropriate selection of the studies under examination.

FUNDING

This work was supported by grants from Secretaría de Ciencia y Técnica, Universidad Nacional de Córdoba (SECyT; 2017-2018) and Secretaría de Políticas Integrales sobre Drogas de la Nación Argentina (SEDRONAR 2018-2019) awarded to JM.

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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.

frontiers in Behavioral Neuroscience

MINI REVIEW
published: 31 March 2020
doi: 10.3389/fnbeh.2020.00045

Adolescent Ethanol Exposure: Anxiety-Like Behavioral Alterations, Ethanol Intake, and Sensitivity

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Adolescence is a developmental period associated with rapid age-specific physiological, neural, and hormonal changes. Behaviorally, human adolescents are characterized by age-typical increases in novelty-seeking and risk-taking, including the frequent initiation of alcohol and drug use. Alcohol use typically begins during early adolescence, and older adolescents often report high levels of alcohol consumption, commonly referred to as high-intensity drinking. Early-onset and heavy drinking during adolescence are associated with an increased risk of developing alcohol use disorders later in life. Yet, long-term behavioral consequences of adolescent alcohol use that might contribute to excessive drinking in adulthood are still not well understood. Recent animal research, however, using different exposure regimens and routes of ethanol administration, has made substantial progress in identifying the consequences of adolescent ethanol exposure that last into adulthood. Alterations associated with adolescent ethanol exposure include increases in anxiety-like behavior, impulsivity, risk-taking, and ethanol intake, although the observed alterations differ as a function of exposure regimens and routes of ethanol administration. Rodent studies have also shown that adolescent ethanol exposure produces alterations in sensitivity to ethanol, with these alterations reminiscent of adolescent-typical ethanol responsiveness. The goal of this mini-review article is to summarize the current state of animal research, focusing on the long-term consequences related to adolescent ethanol exposure, with a special emphasis on the behavioral alterations and changes to ethanol sensitivity that can foster high levels of drinking in adulthood.

Keywords: adolescence, alcohol, adolescent ethanol exposure, anxiety, ethanol intake, ethanol sensitivity

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *27 November 2019*
Accepted: *16 March 2020*
Published: *31 March 2020*

Citation:
Towner TT and Varlinskaya EI (2020) Adolescent Ethanol Exposure: Anxiety-Like Behavioral Alterations, Ethanol Intake, and Sensitivity. Front. Behav. Neurosci. 14:45. doi: 10.3389/fnbeh.2020.00045

INTRODUCTION

Initiation of alcohol use is commonly reported during early (11–15 years of age) adolescence (Faden, 2006; Masten et al., 2009; Morean et al., 2018), and this early initiation is frequently associated with the development of alcohol abuse/dependence later in life (Kuntsche et al., 2016). Several researchers have reported that adolescents who begin drinking at or before the age of 14 are at an elevated risk of becoming alcohol-dependent compared to those who initiate alcohol use at the age of 19 or later (DeWit et al., 2000; Ehlers et al., 2006; Dawson et al., 2008). Likewise, a fast progression from first drink to the first intoxication is a strong predictor and indicator of binge and high-intensity drinking among adolescents (Morean et al., 2014, 2018; Kuntsche et al., 2016; Patrick et al., 2019).

The developing adolescent brain is thought to be particularly vulnerable to alcohol (Olsson et al., 2016; Silveri et al., 2016; Spear, 2018). Thus, early initiation of alcohol use together with high levels of drinking during adolescence (Patrick et al., 2013) can potentially disrupt maturational changes occurring in the brain (Blakemore, 2012; Mills et al., 2014). Therefore, investigations of the consequences of ethanol exposure on the adolescent brain and behavior are critical for understanding the relationship between adolescent drinking and the development of alcohol use disorders later in life.

Rodent models of adolescence allow researchers to determine the consequences of adolescent ethanol exposure that may contribute to the development of alcohol use disorders in adulthood. Behaviorally, consequences of adolescent alcohol exposure include reductions in cognitive flexibility, as well as increases in risk-taking and anxiety, which are associated with multiple neural alterations reviewed in several recent publications (Pascual et al., 2009; Crews et al., 2016, 2019; Spear, 2018). The focus of this mini-review article is on the specific consequences of adolescent alcohol exposure that might foster high levels of ethanol intake later in life and, therefore, become risk factors for the development of alcohol use disorders.

ADOLESCENT ETHANOL EXPOSURE: IMPACT ON ETHANOL INTAKE IN ADULTHOOD

Different animal models of ethanol exposure have been used to determine whether adolescent experience with ethanol influences subsequent intake. Voluntary ethanol consumption in laboratory rodents is commonly assessed using a two-bottle choice (2BC) paradigm, in which animals are given free access to water and ethanol, with this paradigm also used for adolescent ethanol exposure. Intake levels in the 2BC paradigm generally do not produce blood ethanol concentrations (BECs) in the binge range (80 mg/dl and higher), but rather in the low-to-moderate range. The original 2BC paradigm has been modified to increase ethanol consumption, *via* models such as the drinking in the dark (DID) paradigm (reviewed in Thiele and Navarro, 2014) and scheduled high-alcohol consumption (SHAC) procedure (Finn et al., 2005). These modified voluntary consumption paradigms implement a restricted time of access to ethanol that allows for elevated ethanol intake resulting in higher BECs, similar to those achieved by forced exposure. Indeed, studies focused on ethanol exposures that produce BECs well into the binge range (100–250 mg/dl) use forced ethanol administration *via* intragastric gavage (IG), intraperitoneal injections (IP), and ethanol vapor inhalation (VI). Findings from studies evaluating the effects of adolescent ethanol exposure on later ethanol intake are presented in **Table 1**.

More than half of the studies outlined in **Table 1** demonstrate that adolescent ethanol exposure results in persistent increases in ethanol intake in adulthood, findings across multiple models of exposure and intake assessment methods. For example, although Blomeyer et al. (2013) exposed male, Wistar rats *via* a 2BC paradigm during mid-adolescence, and Pandey et al. (2015) administered ethanol IP (2 g/kg) in male, Sprague–Dawley rats, both studies demonstrated higher intakes in ethanol-exposed animals relative to their age-matched controls. Similarly, Gass et al. (2014) reported increased ethanol intake in adulthood following adolescent VI exposure. Some studies have also shown increased intake in adulthood following a combination of voluntary (2BC) and forced (VI) ethanol exposures (Criado and Ehlers, 2013; Amodeo et al., 2018). However, 19 of the studies reviewed found either no effects of adolescent ethanol exposure, more pronounced effects of adult exposure on ethanol intake, or an equivalent response following adolescent and adult exposures (see **Table 1**).

Several factors potentially contribute to these inconsistent findings, with exposure duration and exposure timing representing two important variables that vary drastically between the different studies. Throughout the reviewed literature, exposure duration ranged from 4 days (Jacobsen et al., 2018) to as long as 8 weeks (Fullgrabe et al., 2007) during adolescence and in some cases well into adulthood (Fernandez et al., 2016). The results of the only study that directly addressed the issue of exposure timing within the adolescent period on ethanol intake later in life demonstrated that early adolescent males (postnatal days 30–43) are more vulnerable to ethanol-exposure-related increases in ethanol intake than their more mature (postnatal days 45–58) adolescent counterparts (Alaux-Cantin et al., 2013).

Only a third of the studies (5 out of 16) that used voluntary ethanol consumption, such as 2BC or similar models of adolescent exposure, demonstrated increased ethanol intake later in life. Very few studies reported BEC data during the voluntary consumption exposure phase, and those that did generally observed low BECs that ranged from 0 to 100 mg/dl with averages around 25–35 mg/dl (Gilpin et al., 2012; Broadwater et al., 2013; Amodeo et al., 2017; however see O'Tousa et al., 2013). However, findings of studies implementing voluntary consumption in which higher BECs (80–200 mg/dl) were achieved, commonly from DID and SHAC procedures, generally supported elevated intake levels in adulthood. Of the studies that report increased ethanol intake in adulthood, forced adolescent exposure appears to more effectively increase ethanol intake than voluntary exposure models. Indeed, more than half (11 out of 19) of the studies that employed forced exposure paradigms reported exposure-related increases in ethanol intake. Together, these findings suggest that to enhance ethanol intake later in life, adolescent exposure models, both voluntary and forced, should produce BECs well into the binge range.

Many studies that reported increases in ethanol intake following adolescent ethanol exposure did not include other age groups for comparison. Of the studies that included adolescents and adults, previous exposure to ethanol tended to increased ethanol intake later in life regardless of exposure timing (Hefner and Holmes, 2007; Tambour et al., 2008; Strong et al., 2010; Carrara-Nascimento et al., 2013; O'Tousa et al., 2013; Amodeo et al., 2017) or following adult exposure only (Fullgrabe et al., 2007; Jury et al., 2017), suggesting that exposure-related increases in ethanol

TABLE 1 | Effects of adolescent ethanol exposure on ethanol intake.

Strain	Sex	Exposure			Test	Results	Reference
		Route and Dose	Pattern	Timing and Duration (days)			
Sprague–Dawley rats	Male	IP 3.0 g/kg	Intermittent	P30–43 (14) P45–58 (14)	2BC Operant SA	**Increased intake** after P30–43 exposure	Alaux-Cantin et al. (2013)
	Male	IP 4.0 g/kg	Intermittent	P24–33 (10) P69–78 (10)	2BC	No change in intake	Broadwater et al. (2011)
	Male Female	1 bottle with 10% EtOH in SS	Intermittent	P28–42 (15)	30-min access to 10% sweet EtOH	**Increased intake** of a familiar solution during initial sessions	Broadwater et al. (2013)
	Male	Single bottle with fade on to 20% EtOH	Continuous	P35–250 (215) P75–290 (215)	Continuous single bottle access	**Increased intake** for adolescent-onset animals	Fernandez et al. (2016)
	Male Female	IG 1.5, 3.0, or 5.0 g/kg	Intermittent	P28–45 (18)	2BC	**Increased intake**	Maldonado-Devincci et al. (2010)
	Male	IP 2.0 g/kg	Intermittent	P28–41 (14)	2BC	**Increased intake**	Pandey et al. (2015)
	Male	IP 2.0 g/kg	Intermittent	P28–41 (14)	2BC	**Increased intake**	Sakharkar et al. (2019)
	Male	VI	Consecutive	P30–39 (10)	Free choice operant	No change in intake	Slawecki and Betancourt (2002)
	Male Female	IG 3.5 g/kg	Intermittent	P25–45 (21)	Social drinking	No change in intake	Varlinskaya et al. (2017)
	Male	2BC 10% EtOH	Continuous	P27–90 (64) P70–90 (20)	2BC	No change in intake	Vetter et al. (2007)
	not stated	2BC 5% EtOH sweetened	Variable	P22–50 (29)	Operant SA	No change in intake	Williams et al. (2018)
Wistar rats	Male	2BC 20% EtOH	Intermittent	P26–59 (34) P92–125 (34)	Operant SA	Increased SA regardless of exposure age	Amodeo et al. (2017)
	Male Female	2BC 20% EtOH + VI	Variable	P22–67 (46)	2BC	**Increased intake**	Amodeo et al. (2018)
	Male	1 bottle 5% EtOH	Intermittent	P40–90 (51)	2BC	**Increased intake**	Blomeyer et al. (2013)
	Male	2BC 10% EtOH + VI	Variable	P29–100 (72)	2BC	**Increased intake**	Criado and Ehlers (2013)
	Female	3BC 5 and 20% EtOH	Continuous	P31–87 (56) P71–127 (56)	3BC	Adult onset consumed more ethanol than adolescent onset	Fullgrabe et al. (2007)
	Male Female	4BC 5, 10, and 20% EtOH	Continuous	P19–28 (10) P28–37 (10) P90–99 (10)	4BC	No difference in intake	García-Burgos et al. (2009)
	Male	1 bottle of 5% sweetened EtOH Operant SA IP 2 g/kg	Variable	P27–39 (13) P28–42 (14)	Single bottle 30-min access or operant SA	No difference in adult intake following voluntary ethanol consumption; decrease in adult intake following adolescent ethanol exposure *via* injection	Gilpin et al. (2012)

(Continued)

TABLE 1 | Continued

Strain	Sex	Exposure			Test	Results	Reference
		Route and Dose	Pattern	Timing and Duration (days)			
	Male	1 bottle 8% EtOH	Consecutive during the dark phase (12 h/day)	P51–58 (7)	2BC	**Increased intake**	Milivojevic and Covault (2013)
	Male	IP 3.0 g/kg	Intermittent	P25–38 (14)	2BC (24 h and limited access)	**Increased intake**	Pascual et al. (2009)
	Female	2BC 15% EtOH	Continuous	P30–60 (30)	Operant SA	**Increased acquisition**	Rodd-Henricks et al. (2002)
	Male	3BC 5 and 20% EtOH	Continuous	P31–71 (40)	3BC	Adult-onset consumed more ethanol than adolescent-onset	Siegmund et al. (2005)
	Female	2BC 15% EtOH	Continuous	P30–60 (30)	Operant SA	**Increased acquisition**	Toalston et al. (2015)
	Male	Traverse runway to have free access to varying % + yoked controls	Consecutive	P29–54 (25)	2BC	Adolescents that navigated runway to earn ethanol reward had **increased intake** in adulthood compared to yoked controls	Walker and Ehlers (2009)
Long-Evans rats	Male	VI	Intermittent	P28–42 (14)	Operant SA	**Increased intake**	Gass et al. (2014)
	Male	2BC 20% EtOH	Intermittent	P23–56 (24)	2BC	No changes in intake	Moaddab et al. (2017)
	Male	VI	Intermittent	P28–44 (16)	2BC Operant SA	No changes in intake	Nentwig et al. (2019)
	Male Female	1 bottle 5% or 10% EtOH	Continuous	P21–70 (50)	2BC	**Male mice increased intake**; Female mice decreased intake	Siciliano and Smith (2001)
C57 mice	Male	2BC 15% EtOH+ VI	Variable	P30–57 (28) P70–97 (28)	2BC	**Increased intake** but no effect of age of exposure	Carrara-Nascimento et al. (2013)
	Male Female	SHAC 5% EtOH	Limited intermittent	P28–49 (21) P56–77 (21)	2BC	No changes in intake	Cozzoli et al. (2014)
	Male	2BC 10% EtOH	Continuous	P28–49 (21) P42–63 (21)	2BC	No changes in intake	Hefner and Holmes (2007)
	Male	2BC 10% EtOH	Continuous	P24–112 (88) P56–112 (56)	2BC	**Increased intake** with adolescent onset of ethanol consumption	Ho et al. (1989)
	Female	VI	Consecutive	P28–56 (28) P56–84 (28)	2BC	No changes in intake	Jury et al. (2017)
	Male	2BC 15% EtOH+ VI	Variable	P28–70 (42) P56–98 (42)	2BC	No change in intake for adolescent exposed; adult CIE exposed increased intake	Jury et al. (2017)

(Continued)

TABLE 1 | Continued

Strain	Sex	Exposure			Test	Results	Reference
		Route and Dose	Pattern	Timing and Duration (days)			
	Male	4BC-DID 5, 10, 20, and 40% EtOH	Consecutive limited 2-h access	P28–41 (14) P56–69 (14)	DID	**Increased intake** following adolescent ethanol exposure	Lee et al. (2017)
	Male Female	DID 20% EtOH	Consecutive limited 2 h access	P28–42 (14)	DID	**Increased intake**	Moore et al. (2010)
	Male Female	SHAC 5% EtOH	Limited intermittent	P26–47 (21) P58–79 (21)	DID 2BC	No difference in intake during DID; **increased intake** for both sexes during the 2BC following adolescent exposure	Strong et al. (2010)
BALB	Male Female	2BC 10% EtOH; 1 bottle 10% EtOH; Gradual 0.5–10% EtOH	Continuous	P35–84 (49)	2BC	BALB/cByJ **increased intake** regardless of method BALB/cJ **increased intake** following gradual concentration change	Blizard et al. (2004)
	Male Female	IG 2.2 g/kg	Consecutive	P22–25 (4)	DID	**Increased intake**	Jacobsen et al. (2018)
HS/Ibg (HAP2 or WSC1)	Male Female	2BC 10% EtOH	Continuous	P28–42 (14) P60–74 (14)	2BC	**Increased intake** over initial test days but no effect of age of exposure	O'Tousa et al. (2013)
	Male Female	2BC 6% EtOH	Continuous	P28–84 (56) P70–126 (56)	2BC	**Increased intake** but no effect of age of exposure	Tambour et al. (2008)

*EtOH, Ethanol; 2BC, Two bottle choice; 3BC, Three bottle choice; 4BC, Four bottle choice; IP, Intraperitoneal; IG, Intragastric Gavage; VI, Vapor Inhalation; SS, Super sac; DID, Drinking in the Dark; SHAC, Scheduled High Alcohol Consumption; SA, Self-Administration. *Testing took place in adolescence for the adolescent exposed group. The bold text in the table represents results in which increases in ethanol consumption were found.*

consumption may not be specific to adolescent exposure. Therefore, it is still not clear whether adolescents are more vulnerable than adults to ethanol-exposure-related increases in ethanol intake.

The studies conducted to date indicate that many factors contribute to the effects of adolescent ethanol exposure on ethanol intake later in life, including exposure regiments (continuous vs. intermittent), exposure duration, exposure mode (voluntary vs. forced), exposure levels, BECs achieved, and strain. Given their respective effects and contributions, all these factors should be considered in future studies.

ETHANOL SENSITIVITY FOLLOWING ADOLESCENT ETHANOL EXPOSURE

In general, adolescent laboratory rodents are less responsive than their adult counterparts to adverse effects of ethanol that may curb ethanol intake. These adverse effects of ethanol include social inhibition (Varlinskaya and Spear, 2002), sedation (Moy et al., 1998; Silveri and Spear, 1998; Draski et al., 2001), motor impairment (White et al., 2002; Ramirez and Spear, 2010), and aversion (Vetter-O'Hagen et al., 2009; Anderson et al., 2010; Schramm-Sapyta et al., 2014; Saalfield and Spear, 2015, 2019). In contrast, adolescent rats are uniquely responsive to social facilitation induced by low doses of ethanol (Varlinskaya and Spear, 2002, 2007, 2015; Trezza et al., 2009; Willey et al., 2009), with some evidence also suggesting higher responsiveness to the rewarding effects of ethanol during adolescence than in adulthood (Pautassi et al., 2008). Adolescent ethanol exposure produces alterations in responsiveness to ethanol that resemble these adolescent-typical ethanol sensitivities. This retention of adolescent-typical responding to ethanol has been termed as the "lock-in" effect of adolescent ethanol exposure (reviewed in Spear and Swartzwelder, 2014). The "locking in" of adolescent-typical responding to ethanol effects may play a substantial role in increased ethanol intake in adulthood following adolescent exposure.

Effects of chronic adolescent exposure to ethanol on ethanol-induced sedation indexed *via* the loss of the righting reflex (LORR) have been assessed in laboratory rodents. For instance, adult male rats exposed to ethanol during adolescence (P30–48, 1, 2, 3 or 4 g/kg ethanol, IP) and challenged with a hypnotic ethanol dose regained their righting reflex more rapidly than did their non-exposed counterparts (Matthews et al., 2008), with these alterations evident only following high exposure doses of ethanol (3 and 4 g/kg). These results were also replicated by the same group (Matthews et al., 2017) and others using mice (Jury et al., 2017). However, similar decreases in LORR duration were evident following adult exposure as well (Jury et al., 2017). These findings suggest that adolescent ethanol exposure results in relative insensitivity to ethanol-induced sedation, although the development of metabolic tolerance to ethanol cannot be ruled out. Indeed, the development of metabolic tolerance has been reported following adolescent ethanol exposure (Silvers et al., 2003). When adolescent and adult male rats were repeatedly exposed to an ethanol dose of 4 g/kg and challenged with the same dose 24 h after the last exposure, adult rats, but not their adolescent counterparts, demonstrated chronic tolerance to the sedative effects of ethanol that appeared to be metabolic, but not functional (Broadwater et al., 2011). Evidence of decreased sensitivity to ethanol-induced sedation associated with adolescent ethanol exposure came from the study of Quoilin et al. (2012): female Swiss mice exposed to ethanol during adolescence regained the righting reflex at higher BECs than controls.

Adult animals exposed to ethanol during adolescence become relatively insensitive to the aversive effects of ethanol assessed *via* ethanol-induced conditioned taste aversion (CTA). Diaz-Granados and Graham (2007) exposed adolescent male mice to ethanol vapor either continuously or intermittently and found attenuated CTA to ethanol later in life, with intermittent exposure producing greater attenuation and adult exposure not producing similar effects. Saalfield and Spear (2015), assessing the impact of ethanol exposure (4.0 g/kg, IG) during early (P25–45) and late (P45–65) adolescence on ethanol-induced CTA in male rats, found that both adolescent exposures resulted in decreased sensitivity to the aversive effects of ethanol. Alaux-Cantin et al. (2013) also found that male rats exposed to ethanol during early adolescence (3 g/kg, IP, P30–43) demonstrated attenuated ethanol-induced CTA in adulthood. The reductions in sensitivity to ethanol CTA following adolescent ethanol exposure appear to be sex-specific, with only male Long-Evans rats, but not females, demonstrating an attenuated CTA in adulthood following adolescent exposure (Sherrill et al., 2011).

Adolescent ethanol exposure (P25–45, IG, 4 g/kg) of Sprague–Dawley male rats resulted in precipitation of adolescent-typical responding to acute ethanol challenge with social facilitation (i.e., ethanol-induced increases in peer-directed social behavior) when these males were tested in adulthood (Varlinskaya et al., 2014). Enhanced sensitivity to ethanol reinforcement indexed *via* a significant leftward shift in the dose-response curve for ethanol self-administration into the posterior ventral tegmental area following adolescent ethanol exposure (4 g/kg IG, P28–48) was also evident in adult male and female Wistar rats, as well as in alcohol-preferring (P) male rats (Hauser et al., 2019). Carrara-Nascimento et al. (2014) showed that adult male Swiss mice exposed to ethanol during adolescence displayed a robust CPP to 2.0 g/kg ethanol, whereas adult exposure decreased sensitivity to the reinforcing properties of ethanol. Similarly, BALB/c adult mice demonstrated enhanced sensitivity to ethanol-induced CPP following only four exposures to ethanol given during the juvenile period on P22–25 (Jacobsen et al., 2018).

Taken together, the experimental findings demonstrate that exposure to ethanol during adolescence changes sensitivity to many ethanol effects later in life, decreasing sensitivity to adverse effects of ethanol and making adult laboratory rodents more sensitive to stimulatory and rewarding properties of ethanol. This pattern of sensitivity to the adverse and desired ethanol effects, reminiscent of that typically shown by adolescent rodents, may allow adult animals

to ingest higher amounts of ethanol without experiencing negative consequences.

ADOLESCENT ALCOHOL EXPOSURE: ANXIETY-LIKE BEHAVIORAL ALTERATIONS

Adolescents and young adults who engage in problematic drinking often drink for enhancement of positive emotional states or alleviation of negative affective states (Ham and Hope, 2003; Kuntsche et al., 2006). The association between negative reinforcement and alcohol use has been shown to become stronger in individuals with alcohol use disorder, with no changes evident in the association between positive reinforcement and alcohol consumption (Cho et al., 2019). Indeed, available research suggests relatively strong associations between adolescent alcohol use and increased prevalence of anxiety and depression disorders in adulthood (Rohde et al., 2001; Jeanblanc, 2015). In older adults, alcohol use disorder is frequently comorbid with depression and anxiety (Vorspan et al., 2015; Wiener et al., 2018). Therefore, the assessment of affective behavioral alterations in animal models of adolescent alcohol exposure seems utterly important.

Increases in anxiety-like behavior have been reported using different models of adolescent ethanol exposure. For instance, adult male Sprague–Dawley rats exposed to 2 g/kg ethanol (Kokare et al., 2017; Kyzar et al., 2017, 2019; Sakharkar et al., 2019) or 4 g/kg ethanol (Van Skike et al., 2015) given IP during adolescence, as well as male Long-Evans rats exposed IG to a 1.5 g/kg ethanol dose (Loxton and Canales, 2017), demonstrated elevated levels of anxiety-like behavior when tested on the elevated plus-maze (EPM). Our recent findings indicated that IG ethanol exposure of Sprague–Dawley males and females during early/mid-adolescence (P25–45) results in enhanced anxiety-like behavior on the EPM in adulthood regardless of sex (Varlinskaya et al., 2019). However, only males demonstrated enhanced anxiety-like behavior on the EPM following late-adolescent/emerging-adulthood exposure (P45–65), suggesting that the effects of ethanol exposure are sex- and exposure-timing dependent.

However, Torcaso et al. (2017), exposing male Wistar rats IG to ethanol (3 g/kg, P37–44) reported no behavioral changes on the EPM, whereas other researchers have demonstrated that adolescent ethanol exposure resulted in decreases of anxiety-like behavior on the EPM (Gilpin et al., 2012; Gass et al., 2014). For example, Long-Evans male rats were intermittently exposed to ethanol *via* VI during early-mid adolescence (P28–P42), and this exposure regimen resulted in decreased anxiety, as indexed by increased open arm behavior evident in adult rats (Gass et al., 2014). Similarly, adolescent (P28–P42) ethanol exposure of male Wistar rats *via* self-administration increased percent open arm time on the EPM when these males were tested in adulthood (Gilpin et al., 2012). These inconsistent results are likely associated with procedural differences such as rat strain, route of ethanol administration, phase of light/dark cycle during testing, and pre-test manipulations (see Hogg, 1996; Carobrez and Bertoglio, 2005). For example, the two studies that reported decreased anxiety-like behavior on the EPM tested animals in low-light conditions during the dark part of the light/dark cycle, conditions that may reduce anxiety-like behavior (Gilpin et al., 2012; Gass et al., 2014). It is possible that the observed increases in open arm entries and/or open arm time reflect disinhibition but not decreases in anxiety-like behavior, since the characteristics of the test situation determine whether anxiety or disinhibition are manifested in the EPM (Ennaceur, 2014).

Anxiety-like alterations associated with adolescent ethanol exposure were reported for other tests of anxiety as well. Experiments using the light/dark box have shown that adolescent ethanol exposure increases time spent in the dark portion of the apparatus and decreases entries into the light side in male Sprague–Dawley rats (Pandey et al., 2015; Vetreno et al., 2016; Sakharkar et al., 2019). Anxiety-like behavior in the open-field (Coleman et al., 2014; Yan et al., 2015) and marble-burying test (Lee et al., 2017) have also been enhanced following adolescent ethanol exposure of Sprague–Dawley males (Yan et al., 2015) and male C57BL/6J mice (Coleman et al., 2014; Lee et al., 2017). When tested in adulthood, male Sprague–Dawley rats exposed to ethanol during early-mid adolescence demonstrated social anxiety-like behavioral alterations indexed *via* decreases in social investigation and social preference (Varlinskaya et al., 2014), with no changes in social behavior evident following late adolescent ethanol exposure.

Increased anxiety-like behavior following adolescent ethanol exposure may also contribute to increases in ethanol intake due to the anxiolytic properties of ethanol. Although links between anxiety and alcohol consumption have been commonly reported in humans (Vorspan et al., 2015) and laboratory rodents (Pelloux et al., 2015), it remains to be investigated whether animals that demonstrate increases in anxiety following adolescent ethanol exposure drink more ethanol for its negatively reinforcing, anxiolytic effects.

CONCLUSIONS

Although adolescent alcohol exposure is associated with behavioral alterations and changes in ethanol sensitivity, it is still not clear whether these alterations contribute to increases in ethanol intake. Considering that very few studies assessing changes in ethanol intake following adolescent ethanol exposure included both sexes, the question of whether responding to ethanol exposure during adolescence differs in males and females remains unanswered. Furthermore, among the studies that assessed changes in ethanol intake, only a limited number included both adolescent and adult ethanol exposure conditions (see **Table 1**), producing mixed results and not allowing to conclude that enhanced ethanol intake in adulthood is specific to adolescent ethanol exposure. The impact of ethanol exposure timing within the adolescent period (i.e., during early vs. late adolescence) on ethanol intake and sensitivity is still not well understood, and this important issue should

also be addressed in future studies (Spear, 2015). The relative insensitivity of adolescents to the acute effects of ethanol is related in part to age differences in compensatory responses, including acute tolerance, that serve to counteract ethanol-induced impairment. Therefore, it is important to investigate whether adolescent ethanol exposure decreases sensitivity to the adverse effects of acute ethanol by enhancing the development of acute tolerance. More studies are needed for a better understanding of the consequences of alcohol exposure during adolescence that might contribute to heavy drinking later in life and put individuals at risk for the development of alcohol use disorders.

AUTHOR CONTRIBUTIONS

TT and EV contributed equally.

FUNDING

The work presented in this manuscript was funded by grants U01 AA019972 (Neurobiology of Adolescent Drinking in Adulthood Consortium—NADIA Project), P50 AA017823 (Developmental Exposure Alcohol Research Center), and T32 AA025606 (Development and Neuroadaptation in Alcohol and Addictions—DNAA Project).

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 09 April 2020
doi: 10.3389/fnbeh.2020.00050

Binge-Like, Naloxone-Sensitive, Voluntary Ethanol Intake at Adolescence Is Greater Than at Adulthood, but Does Not Exacerbate Subsequent Two-Bottle Choice Drinking

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OPEN ACCESS

Edited by:
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Higher Institute of Health (ISS), Italy

Reviewed by:
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University of Palermo, Italy
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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *22 October 2019*
Accepted: *19 March 2020*
Published: *09 April 2020*

Citation:
Salguero A, Suarez A, Luque M, Ruiz-Leyva L, Cendán CM, Morón I and Pautassi RM (2020) Binge-Like, Naloxone-Sensitive, Voluntary Ethanol Intake at Adolescence Is Greater Than at Adulthood, but Does Not Exacerbate Subsequent Two-Bottle Choice Drinking. Front. Behav. Neurosci. 14:50. doi: 10.3389/fnbeh.2020.00050

The present study assessed the effects of ethanol exposure during adolescence or adulthood. We exposed Wistar rats, males or females, to self-administered 8–10% (v/v) ethanol (BINGE group) during the first 2 h of the dark cycle, three times a week (Monday, Wednesday, and Friday) during postnatal days (PDs) 32–54 or 72–94 (adolescent and adults, respectively). During this period, controls were only handled, and a third (IP) condition was given ethanol intraperitoneal administrations, three times a week (Monday, Wednesday, and Friday), at doses that matched those self-administered by the BINGE group. The rats were tested for ethanol intake and preference in a two-bottle (24 h long) choice test, shortly before (PD 30 or 70) and shortly after (PD 56 or 96) exposure to the binge or intraperitoneal protocol; and then tested for free-choice drinking during late adulthood (PDs 120–139) in intermittent two-bottle intake tests. Binge drinking was significantly greater in adolescents vs. adults, and was blocked by naloxone (5.0 mg/kg) administered immediately before the binge session. Mean blood ethanol levels (mg/dl) at termination of binge session 3 were 60.82 ± 22.39. Ethanol exposure at adolescence, but not at adulthood, significantly reduced exploration of an open field-like chamber and significantly increased shelter-seeking behavior in the multivariate concentric square field. The rats that had been initially exposed to ethanol at adolescence drank, during the intake tests conducted at adulthood, significantly more than those that had their first experience with ethanol at adulthood, an effect that was similar among BINGE, IP and control groups. The study indicates that binge ethanol drinking is greater in adolescent that in adults and is associated with heightened ethanol intake at adulthood. Preventing alcohol access to adolescents should reduce the likelihood of problematic alcohol use or alcohol-related consequences.

Keywords: ethanol, Wistar, binge exposure, naloxone, adolescence

INTRODUCTION

Alcohol (subsequently also referred to as ethanol in the context of pre-clinical studies) use is highly prevalent during adolescence in most western countries. Illustrating this point, a study (Pilatti et al., 2017) reported that, in the 6 months preceding data collection, 70% of a sample of college students from Argentina (n > 4,000, average age: 19 years) ingested 6–7 drinks of alcohol on the same drinking occasion; and 55% consumed 4–5 drinks in 2≤ h (a pattern known as "binge drinking"). Approximately 33 and 20% reported these patterns, respectively, on a weekly basis, and the average consumption on Saturdays was 5–8 drinks, reaching 10 in those at-risk for exhibiting family history of alcohol problems. These consumption patterns can yield immediate negative consequences [domestic accidents, increased risk of engaging in interpersonal violence or in unsafe sexual practices (Pilatti et al., 2014; Wicki et al., 2018)], but they are also associated with heightened risk of developing alcohol use disorders later in life (Pedersen and Skrondal, 1998; Hingson and Zha, 2009). This is, the earlier the first experience with alcohol, the greater the odds of high-risk alcohol consumption (Rial Boubeta et al., 2018) or alcohol use disorders, although it not yet clear if these two events are causally related, or if they are dependent on a third factor [e.g., genetic predisposition (Buchmann et al., 2009)].

This "early debut" effect (i.e., the early the onset of alcohol use, the greater the later problematic use of alcohol) has been shown across cultures, and is illustrated by a seminal clinical work (DeWit et al., 2000), that reported 16% of alcohol dependence in those who began drinking at 11–12 years, but only 1% in those who started at age 19. Our studies (Pilatti et al., 2013) have also indicated that college students who started drinking alcohol at ≤15 years exhibit significantly more alcohol use and drunkenness than those who had their first contact with the drug after age 15. Perhaps more important, another clinical study has recently shown (Vera et al., 2019) that the initial contact with alcohol is not as relevant, as a predictive milestone for subsequent problematic substance use, as the first intoxication or drunkenness episode. In the latter work a greater number of alcohol-related negative consequences after an early onset of drinking was observed only in participants that already had experienced an intoxication or drunkenness episode. Level of alcohol-related problems was low and not affected by age of drinking onset, in those that were drunkenness naïve. Another study (Kuntsche et al., 2013) indicated that early drunkenness, but not early drinking, predicted several adolescent problem behaviors. This, together with the high prevalence of binge drinking in adolescents, suggests that pre-clinical models of "early alcohol initiation" should focus not so much on mere adolescent drug exposure, but on a type of exposure akin to that of drunkenness. Modeling drunkenness in rats or mice is problematic, as drunkenness is mainly defined by a subjective state. A better alternative is to generate models that induce high levels of ethanol consumption in a short timeframe, compatible with the definition of binge drinking.

The pre-clinical models of the "early alcohol debut" effect have not been consistent as to whether early onset of ethanol use is associated with later heightened ethanol use. The phenomenon has been modeled in rodents by repeatedly exposing rats or mice to high doses of i.p. or i.g. ethanol (e.g., 2.5–3.0 g/kg every 48 h) during the adolescence, which in rodents is usually defined as the period between postnatal days (PD) 28–42 or 60 (Spear, 2000). These experimenter-administered treatments induce, in mice or rats, neuroinflammation (Fernandez-Lizarbe et al., 2009; Pascual et al., 2009), alterations in glutamatergic and dopaminergic transmission (Trantham-Davidson et al., 2017), and also alter the neural pruning of the latter transmitter system (Pascual et al., 2009). They also exert detrimental effects upon the functionality of the hypothalamic-pituitary-adrenal axis (Asimes et al., 2017) and modulate gene expression of histone deacetylase 1 (Lopez-Moreno et al., 2015). These studies are useful to demonstrate persistent changes associated with early exposure to alcohol that induces level of intoxication compatible with or beyond the level that define binge drinking, but they have the limitation of employing routes of administration that exert behavioral and neural effects very different from those induced by the self-administration of the drug. It is not surprising, then, that these models have generated conflicting data. In our laboratory (Fabio et al., 2014) we have observed that only two or five ethanol intubations during the early adolescence of the rat increase the subsequent consumption of ethanol, while the same treatment in adulthood does not exert significant effects, data consistent with that reported by Pascual et al. (2009). However, others did not observe alterations in adult ethanol consumption after forced exposure to vaporized ethanol during the adolescence of the rat (Nentwig et al., 2019). Illustrating the contradictions inherent in these models, both Broadwater et al. (2011) and our laboratory (Pautassi et al., 2015) observed that 6 or 10 i.p. or i.g. administrations of ethanol (2.25–4.0 g/kg every 24 or 48 h) induced chronic tolerance to ethanol or inhibited aversive conditioning toward alcohol in adolescent, but not in adult, rats. However, both studies (Broadwater et al., 2011; Pautassi et al., 2015) reported that the adolescents exposed to ethanol exhibited similar levels of ethanol intake, when evaluated in two-bottle choice tests, than adolescents that had been only exposed to vehicle.

The present study assessed the effects of ethanol exposure during adolescence or young adulthood on two-bottle, free-choice, ethanol drinking. The latter consumption was tested either immediately after termination of ethanol exposure, or in late adulthood, at PDs 120–139. Specifically, we assessed short- and long-term effects of early or late age of onset of alcohol drinking, in an animal model – adapted from the drinking-in-the-dark (DID) paradigm (Boehm et al., 2008; Rodriguez-Ortega et al., 2019) – that mimics a binge pattern of alcohol consumption. In other words, the aim was not just to model early onset of drinking, but to induce levels of drinking akin to those found during a binge drinking episode, whose threshold is often defined at 80 mg/dl (e.g., Hosova and Spear, 2017).

More in detail, adult – male or female – rats self-administered 8–10% (v/v) ethanol during the first 2 h of the dark cycle, three times a week for 4 weeks. Controls were only handled and a third condition was given ethanol i.p. administrations, three times a week for 4 weeks, at doses that matched those

self-administered. The rats were tested for ethanol intake and preference in a two-bottle (24 h long) choice test, shortly before (i.e., baseline) and shortly after exposure to the binge protocol; and then were tested for 3 weeks during late adulthood (PDs 120–139) in intermittent two-bottle ethanol intake tests. The effects of the binge-like exposure during adolescence or adulthood upon anxiety response, shelter seeking/risk-taking and recognition memory were also tested, via the light-dark box (LDB) test (Acevedo et al., 2014), the multivariate concentric square field (MSCF) test (Roman and Colombo, 2009; Ekmark-Lewen et al., 2010) and the novel object recognition (NOR) test (Antunes and Biala, 2012), respectively. Experiment 2 assessed if treatment with the opioid antagonist naloxone could inhibit ethanol binge drinking at adolescence, and measured the blood ethanol levels (BELs) achieved during the binge procedure.

MATERIALS AND METHODS

Experimental Design and Subjects

A total of 155 Wistar rats (129 in Experiment 1, 26 in Experiment 2) were employed. A 2 (age of first contact with ethanol: adulthood vs. adolescence) × 2 (sex: male vs. female) × 3 (mode of early ethanol exposure: binge-like self-administration, i.p. administrations or control, BINGE, IP and CONTROL groups, respectively) factorial was employed in Experiment 1, with 8–12 Wistar rats in each group. Experiment 2a assessed the effects of naloxone upon binge ethanol drinking in 10 male rats, whereas another group of 10 males was administered vehicle. Experiment 2b exposed 6 male adolescents to three sessions of binge drinking. These rats, which were not administered naloxone or vehicle, were sacrificed at the end of the third binge session. Blood samples were obtained and subsequently processed to yield a measure of BELs achieved during the 2 h binge session. A schematic representation of the experiments, containing a detailed account of the number of animals in each experimental group can be found in **Figure 1**.

The rats were born and reared at one of the vivarium of the Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET-UNC; Córdoba, Argentina), a producer of specific pathogen free rats, and were derived from 20 dams. As per policy of the vivarium, all the litters are culled on PD1 to 10 rats (5 males, 5 females, whenever possible). Lights were turned on at 645 and turned off at 1845. Litter effects were controlled by not including more than one male and one female from each litter to each group. Weaning was performed at PD 21, and from that on the rats were housed in same-sex pairs. The procedures complied with the Declaration of Helsinki, the ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals promulgated by the NIH and the EU. The procedures were certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC.

Repeated Exposure to Ethanol on Adolescence or Early Adulthood (Exp. 1)

The rats, males and females, were housed into same-sex groups of two and weighed every day. The day before each BINGE session or IP administration the rats in groups BINGE and IP were given 50% of the water they usually consumed, as a means to promote ethanol consumption in the upcoming binge session or, in the case of the IP group, to keep hydration conditions similar to those in the BINGE group.

For 4 weeks (Adolescents: PDs 32–54, Adults: PDs 72–94) the BINGE rats were exposed on Mondays, Wednesdays and Fridays (except the first Monday of the 1st week and the last Friday of the 4th week, see next section) to a bottle of 8% (first two sessions) or 10% ethanol (third and subsequent session) between 1900 and 2100 h.; this is, 15 min after the beginning of the dark cycle. Subsequently, the rats in the IP group were given an intraperitoneal administration of ethanol (20% v/v, volume of administration: 0.01 ml/g of body weight) whose dose was matched for the level of ethanol ingestion exhibited by their same-sex counterparts of the binge group. This is, the rats in the IP group were exposed to ethanol three times a week for 4 weeks, mirroring the schedule of ethanol exposure of the BINGE group. The inclusion of the IP group, which could be referred to as a "matched condition," was meant to determine whether any effect of early alcohol exposure upon later ethanol drinking during late adulthood was a consequence of total ethanol exposure or if the mode of exposure (self- vs. experimenter-administered) was the key factor. Control rats were left undisturbed, except for the daily weighing procedure. Other than in the times specified, BINGE and IP rats were given water ad-libitum.

The binge protocol was designed by combining well-established preclinical protocols from our group (Wille-Bille et al., 2017) to assess every-other-day ethanol drinking and those of the daily limited-access ethanol intake model referred to as DID (Boehm et al., 2008). At the beginning of each binge session the housing chambers were divided into two sections, by a Plexiglas separator, and each animal occupied half of the cage. Thus, rats in the BINGE group could smell (but not touch) each other, reducing potential isolation effects. Each section was equipped with one glass bottle, with rubber caps with stainless a steel spout with round tip. The rats were exposed to a bottle filled with 8% or 10% alcohol (vehicle: tap water). Spillage/leaking was accounted for by having a control bottle in an empty cage. The rats had *ad libitum* access to food during the 2 h session. After the 2 h (that is, at 2100) the bottle was replaced by a water bottle. The ethanol bottle was weighed before and after the session and these scores were used to calculate the g/kg of alcohol consumed.

Two-Bottle Choice Ethanol Intake Tests Conducted Immediately Before or Immediately After Binge Exposure (Exp. 1)

One of the aims of the study was to evaluate free-choice ethanol drinking immediately after termination of the chronic, binge-like ethanol exposure. Thus, the rats – males and females – were assessed on a two-bottle, 24 h free-choice, test on PD 30 or 70 (adolescent or adult groups, baseline pre-test before any treatment) and on PD 56 or 96 (i.e., post-test after termination of the binge-like exposure or the intraperitoneal administrations).

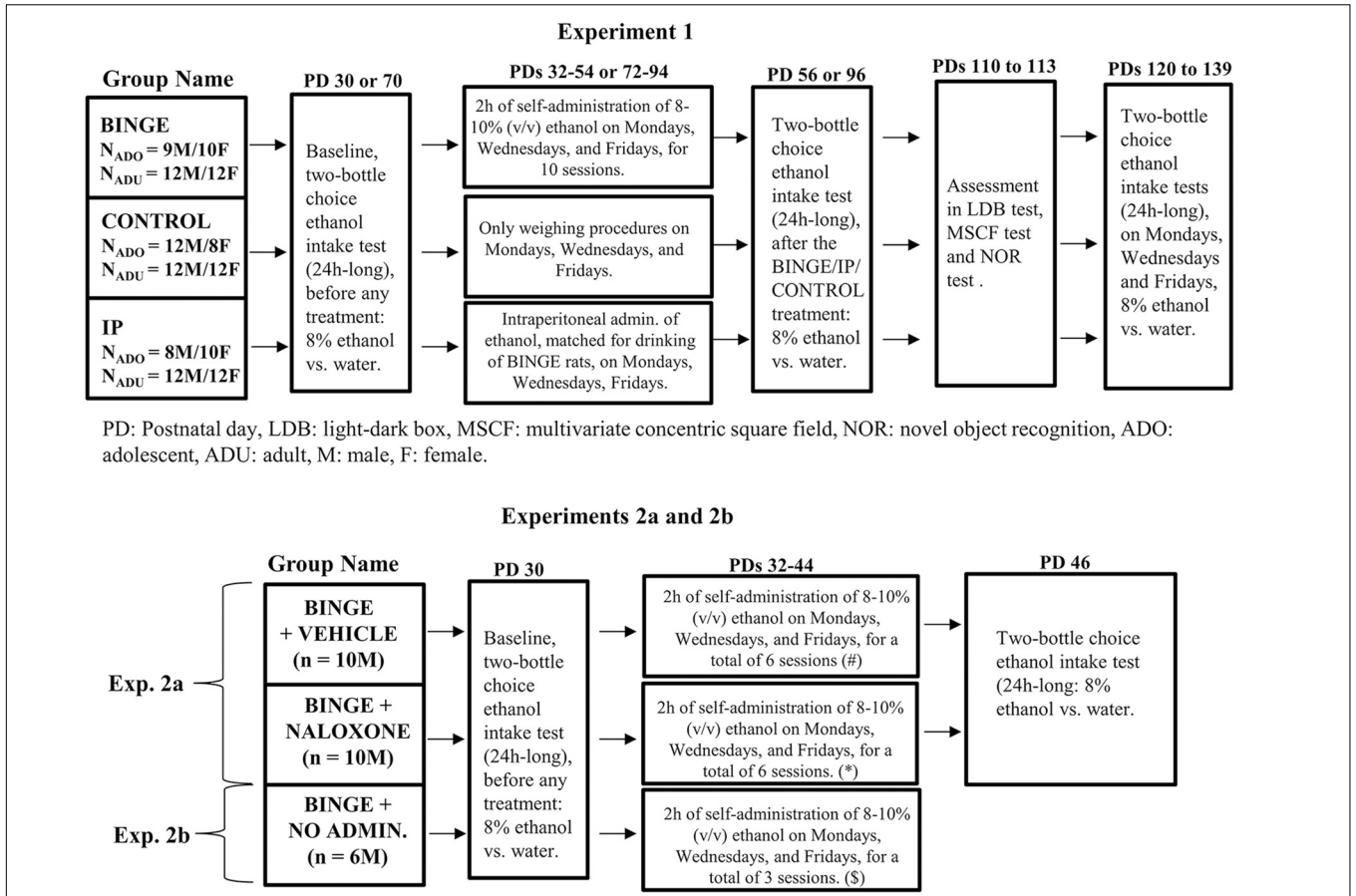

FIGURE 1 | Schematic representation, including experimental timelines and sample size in each group, of the methods for the analysis of short- and long-term effects of ethanol binge drinking, in Experiment 1 and in Experiments 2a and 2b (upper and lower sections, respectively). In Experiment 1 the adolescent or adult rats, males or females, self-administered 8–10% (v/v) ethanol during the first 2 h of the dark cycle, three times a week for 4 weeks (BINGE group). Rats in the CONTROL group were only handled, and those in the IP condition were given ethanol intraperitoneal administrations, three times a week for 4 weeks, at doses that matched those self-administered by same-sex counterparts in the BINGE condition. The rats were tested for ethanol intake and preference in two-bottle (24 h long) choice tests, shortly before (i.e., baseline) and shortly after exposure to the binge protocol; and then again at late adulthood [postnatal days (PDs) 120–139]. The rats were tested on PDs 110–113 in the light-dark box (LDB) test, the multivariate concentric square field (MSCF) test and the novel object recognition (NOR) test. Experiment 2a repeated the BINGE conditions in male adolescents and assessed if treatment with the opioid antagonist naloxone, administered 30 min before binge sessions 3–6, inhibited ethanol binge drinking at adolescence and later ethanol consumption in a two-bottle choice intake test. Experiment 2b measured the blood ethanol concentrations achieved during the binge procedure, in 6 male rats that were sacrificed at the end of binge session 3.

The two-bottle choice tests were conducted following procedures described previously (Fernandez et al., 2017). Briefly, at 900 a Plexiglas divider was used to individually house each rats in half of the homecage. A special lid allowed equipping each section with two bottles and *ad libitum* access to food. One of the bottles contained water, the other was filled with 8% ethanol (v/v). The bottles were weighed before and after each session, and the difference was used to calculate ethanol intake (g/kg) and the percent preference of ethanol intake [(ethanol consumption/overall fluid consumption) × 100].

Behavioral Assessments Following Exposure to Binge Ethanol (Exp. 1)

At late adulthood all the rats were assessed for anxiety response, shelter-seeking and risk taking and overall exploratory patterns, and for recognition memory. These variables were measured via LDB test (Acevedo et al., 2014), the MSCF test (Roman and Colombo, 2009; Ekmark-Lewen et al., 2010) and the NOR test (Antunes and Biala, 2012), respectively. As it will be described in this section, the MSCF also serves to measure, along with other behaviors, anxiety-like responses. The order of the LDB and the

MSCF tests was randomly counterbalanced and took place on PDs 110 or 111, the NOR took place on PDs 112 and 113.

The LDB test was that described in Wille-Bille et al. (2018). Briefly, we employed a rectangular apparatus featuring two sections [one white (24.5 cm × 25 cm × 25 cm, 400 lux illumination), the other black (17.5 cm × 25 cm × 25 cm, 0 lux illumination)] connected by an opening at floor level. Testing lasted 5 min and began by placing the rats in one of the corners of the white section facing the wall. Time spent in the white compartment, latency (s) to first exit the white compartment and number of transfers between compartments were measured.

The MSCF test (Roman and Colombo, 2009) is a 20-min long assay that allows recording several exploratory behaviors simultaneously (Karlsson and Roman, 2016). The rats are gently introduced in a square-shaped apparatus (48 cm × 48 cm) featuring an open-field like center square (OF, the starting area), a highly illuminated (650 lux) area featuring a ramp (RAMP, 12 cm × 10 cm, 20° incline) that lead to a metallic structure (the BRIDGE, 30 cm × 10 cm, 650 lux) that prompted exploration, a dark and enclosed area that evokes shelter-seeking behavior (SHEL, 0 LUX), 3 connecting corridors or passages (P, 20–30 lux), and a small section similar to the SHELL yet slightly more illuminated (30 lux) and inaccessible by regular horizontal locomotion. Access to the latter area, referred to as challenge area (CHA) required performing a jump through an elevated hole. Time spent in SHELL is usually considered an indicator of anxiety response (Wille-Bille et al., 2018). The test was filmed and time spent and number of entries in each area was recorded offline using JWatcher 1.0 (Blumstein and Daniel, 2007). Total number of entries into the different sections was considered an index of overall motor activity.

The novel object recognition (NOR) test assesses short-term memory (Vogel-Ciernia and Wood, 2014). We employed an open-field like, squared-shaped, arena made of Plexiglas (50cm × 50cm × 50cm), equipped with photo sensors that virtually divided the arena into 25 squares. A software (ITCOMM, Córdoba, Argentina) detected, in a minute-by-minute basis, the number of beam breaks. The rats were introduced into the empty arena for 10 min, in a habituation phase (PD112) that also served to analyze exploratory patterns in an open field like novel environment. A day later (familiarization phase) the rats were left to explore the arena for 5 min, which was now equipped with two identical objects (i.e., A and A', opaque glass flasks) in the upper corners. Twenty minutes later one of the objects was replaced by a new object (B, taller and slightly clearly colored compared to A/A') and the rats had another 5-min trial (testing phase) in which they freely explored the arena. The tests were videotaped and analyzed. Time spent in close proximity to the objects was measured, in a minute-by-minute basis, during the familiarization and testing phase. Time spent in proximity to the new object B at the testing phase was considered an indicator of short-term memory (Vogel-Ciernia and Wood, 2014). The objects were selected on the basis of pilot studies that indicated that rats did not have innate preferences for the objects later designated as familiar or novel.

Two-Bottle Choice Ethanol Intake Tests Conducted at Late Adulthood (Exp. 1)

Another aim of the study was to evaluate the long-term effects of ethanol exposure during adolescence or adulthood, after imposing a relatively long time between the last exposure to binge ethanol, and when both groups of rats (i.e., those with "early" or "late" onset of alcohol use) were equated in terms of age of testing. Therefore, all the rats were tested for ethanol intake for 3 weeks at late adulthood (PDs 120–139) using the two-bottle ethanol intake test described in section " Two-Bottle Choice Ethanol Intake Tests Conducted Immediately Before or Immediately After Binge Exposure (Exp. 1)." These 24 h tests were conducted intermittently, on Mondays, Wednesdays and Fridays, for a total of nine sessions.

Naloxone Administration (Exp. 2a) and Measurement of BELs Achieved During the Binge-Like Protocol (Exp. 2b)

Experiment 2a replicated, in 26 adolescent males, the baseline and post-test two-bottle choice measurements of ethanol intake, and binge exposure sessions, of Exp 1.

Specifically, in Experiment 2a the rats (n = 20) were administered saline 30 min prior to the beginning of binge sessions 1 and 2. From session 3 to session 6, half of the rats received naloxone (Sigma Aldrich, Buenos Aires, Argentina; subcutaneous, 5 mg/5 ml/kg) and the remaining received saline. Naloxone dose and timing of administration were selected based in prior work demonstrating its effectiveness to reduce ethanol self-administration. The six remaining rats were not administered naloxone or vehicle before the binge sessions. At the end of binge session 3 these rats were sacrificed through decapitation and trunk blood samples (2-ml samples) were obtained using a heparinized capillary tube. The samples were centrifuged at high speed (15 min/3,000 rpm) and the vials containing the plasma phase were stored at −70°C for later analysis. BELs were expressed as milligrams of ethanol per deciliter of blood (mg/dL) and assessed via a colorimetric enzymatic method. Specifically, the method was based on the action of the enzyme alcohol dehydrogenase, which uses the oxidized form of the nicotinamide adenine dinucleotide (NAD+) as a cofactor, which is then reduced to NADH. This reduction generates an absorbance increase that is measured at 340 nm. These measurements had a precision/accuracy of ±4 mg/dL and were conducted at LACE labs (Córdoba, Argentina), using a COBAS6000 (Roche, Basel, Switzerland) apparatus.

Statistical Analysis

The variables measured were first checked for normality and homogeneity of variance, to assure the appropriateness of using parametric statistics. Ethanol intake scores (g/kg) during the binge sessions of Experiment 1 were assessed using repeated measures (RM) Analyses of Variance (ANOVAs) that considered age (adolescence, adulthood), and sex (male, female) as between factors and day of assessment (intake sessions 1–10) as the within-measure. Similar RM ANOVAs were used to analyze ethanol intake scores (g/kg and % preference) and water intake

(ml/100 g of body weight) at the two-bottle choice ethanol intake tests conducted before and after the binge sessions. The latter ANOVAs also included mode of ethanol exposure (ethanol exposure via self-administration or i.p. injections, or non-exposed controls: groups BINGE, I.P. and CONTROL, respectively) as a between-subjects factor.

Ethanol intake (g/kg) ingested during the binge protocol of Experiment 2 was analyzed via a 2-way mixed ANOVA, with naloxone administration and day of assessment as the between and within-subject factors, respectively. BELs (mean ± SEM) were correlated with the g/kg ingested on binge session 3 via Pearson's time-moment correlation (i.e., the association considered the BELs measured in the blood samples and the absolute level of ingestion registered in the session that finalized just before the sacrifice).

The variables measured in the LDB test (latency to enter the black section, time spent in the white section and number of transfers) and in the MCSF test (Experiment 1) were analyzed by separate factorial (sex × mode of ethanol exposure × age at first ethanol exposure) ANOVAs. Activity scores (number of beam breaks in the empty arena) during the habituation phase of the NOR protocol were analyzed via RM ANOVAs (age of first ethanol exposure × sex × mode of ethanol exposure; with minutes 1–10 as repeated measure). Behavioral reactivity during the first and second (test) phase of the NOR protocol were analyzed via RM ANOVAs, that considered sex, mode of exposure and age at first ethanol exposure as between factors, whereas time spent in the vicinity of the objects (i.e., A and A' or A and B, first phase and test phase, respectively) was the dependent variable. A relative discrimination index (Di) was also calculated [i.e., time spent exploring the novel object minus time spent exploring the familiar object divided by total exploration time (Lueptow, 2017)] and analyzed via a factorial ANOVAs, that considered sex, mode of exposure and age at first ethanol exposure as between factors. Di scores range between -1 and +1, in which a zero score indicates the lack of preference, a negative score indicates more time spent with the familiar object, and a positive score indicates more time spent with the novel object (Antunes and Biala, 2012).

The significant main effects and significant interactions yielded by the ANOVAs were scrutinized via Tukey's *post hoc* tests or planned comparisons. Planned comparisons were used to analyze significant main effects or interactions comprising between-by-within factors whereas Tukey was used for significant effects involving between-subject factors. There is still debate of which is the best error term for *post hoc* comparisons in significant effects involving within-subject factors. In this scenario, the planned comparisons, which were also employed for a few specific comparisons based on *a-priori* hypotheses, provide a satisfactory compromise between conservativeness and sensitivity (Winer et al., 1991). Data is informed as mean ± SE. Effect sizes of the ANOVAs are described through the partial eta squared ($\eta^2 p$) and the α level was set at ≤0.05. Effect sizes were interpreted as follows: small ($\eta^2 p$ = 0.01–0.05), medium ($\eta^2 p$ = 0.06–0.13), and large ($\eta^2 p$ = ≥ 0.14) (Lakens, 2013). The statistical analyses were conducted with STATISTICA 8.0 (Tulsa, OK, United States).

RESULTS

Ethanol Intake at the Binge-Like, 2 h Sessions of Access to Ethanol (Exp. 1)

As shown in **Figure 2**, level of ethanol ingestion during the 2 h binge sessions was much greater in adolescents than in adults, with 2- to 3-fold differences between these groups. These differences were particularly noticeable in the first testing days and in males. The ANOVA confirmed these impressions. The analysis of absolute ethanol intake (g/kg) revealed significant main effects of Age and Day of Assessment ($F_{1,39}$ = 38.60, $p \leq 0.001$; $\eta^2 p$ = 0.50 and $F_{9,351}$ = 5.87, $p \leq 0.001$; $\eta^2 p$ = 0.13) as well as significant interactions between Sex and Age ($F_{1,39}$ = 6.84, $p \leq 0.05$; $\eta^2 p$ = 0.15), and between Age and Day of Assessment ($F_{9,351}$ = 8.32, $p \leq 0.001$; $\eta^2 p$ = 0.18). The three-way interaction Sex × Age × Day of Assessment also reached significance, $F_{9,351}$ = 2.54, $p \leq 0.01$; $\eta^2 p$ = 0.06. The planned comparisons indicated that ethanol drinking was significantly higher in adolescent than in adult rats; an effect that in males achieved significance in all but the last session, whereas in females was significant in sessions 1, 2, 3, 6, and 10. The adults kept their level of ethanol ingestion stable across the course of the assessment, whereas adolescents exhibited a progressive decrease, with ethanol ingestion in male and female adolescents being significantly greater on Day 1 than on Day 10. In adult rats, ethanol ingestion was significantly greater in females vs. males across most sessions (i.e., sessions 1, 3, 4, 5, 6, 7, 8 and 9), whereas this variable was not affected by sex in adolescents.

Ethanol and Water Intake During the Two-Bottle Choice Ethanol Intake Tests Conducted Before and After Binge Exposure (Exp. 1)

Figure 3 depicts g/kg of ethanol ingested during the two-bottle choice ethanol intake tests conducted before and after binge exposure. The ANOVA revealed significant main effects of Sex and Mode of early ethanol exposure ($F_{1,114}$ = 16.76, $p \leq 0.001$; $\eta^2 p$ = 0.13 and $F_{2,114}$ = 4.39, $p \leq 0.05$; $\eta^2 p$ = 0.07, respectively). Females rats drank more than did their male counterparts. The interaction between Mode of exposure and Day of Assessment was also significant ($F_{2,114}$ = 9.16, $p \leq 0.005$; $\eta^2 p$ = 0.14). The subsequent Tukey's *post hoc* tests indicated that all rats, irrespective of the groups they would be assigned during the binge sessions, ingested similar levels of ethanol during the pre-test. On the contrary, at the post-test the Tukey's *post hoc* tests indicated that the rats that had been binging or had received i.p. administrations of ethanol drank significantly less than CONTROL counterparts.

The ANOVA for ethanol percent preference yielded a pattern (descriptive data not shown) similar to that found for absolute ethanol intake scores. The ANOVA revealed significant main effects of Age and Mode of early ethanol exposure ($F_{1,114}$ = 7.36, $p \leq 0.01$; $\eta^2 p$ = 0.06 and $F_{2,114}$ = 3.32, $p \leq 0.05$; $\eta^2 p$ = 0.06, respectively) and a trend toward a significant effect of Sex, $F_{1,114}$ = 3.19, p = 0.0766; $\eta^2 p$ = 0.03. Adults exhibited greater

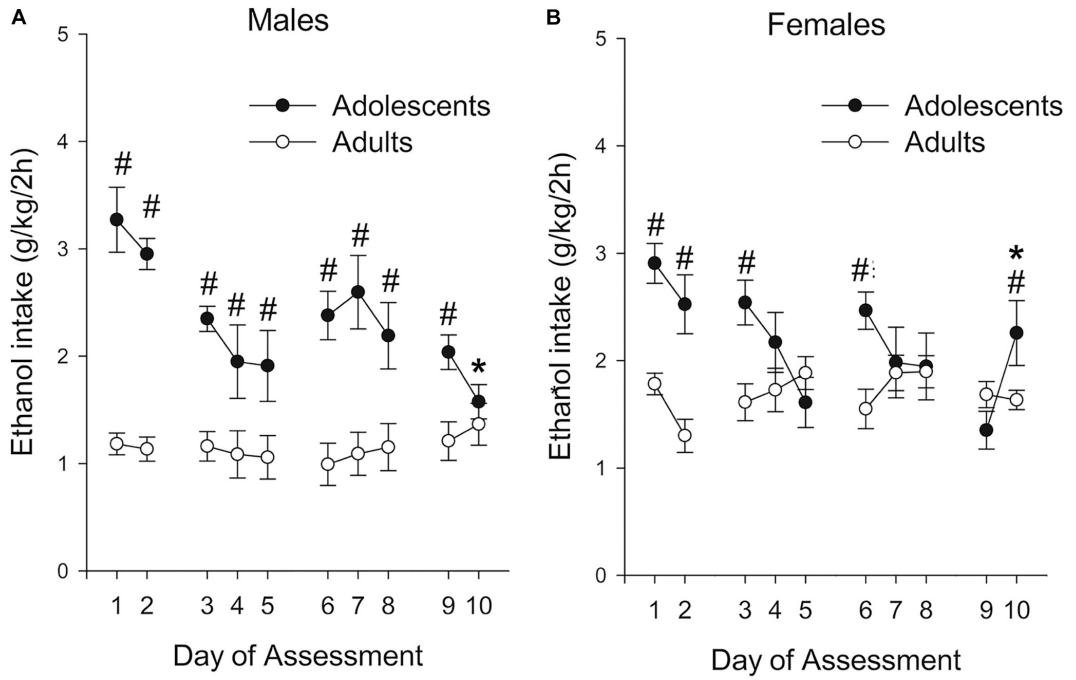

FIGURE 2 | Ethanol intake (g/kg) in male and female Wistar rats (**A** and **B** panels, respectively) as a function of binge intake session (i.e., Day of Assessment 1–10). The rats self-administered 8% (first two sessions) or 10% ethanol (third and subsequent session) during the first 2 h of the dark cycle, three times a week (Monday, Wednesday, and Friday) during postnatal days (PDs) 32–54 or 72–94 (adolescent and adults, respectively). Ethanol drinking was significantly higher in adolescent than in adult rats; an effect that in males achieved significance in all but the last session, whereas in females was significant in sessions 1, 2, 3, 6, and 10. These significant differences are indicated by the numeral (#) sign. Ethanol ingestion in male and female adolescents was significantly greater on Day 1 than on Day 10, an effect indicated by the asterisk (*) sign. Nineteen adolescent (9 males, 10 females) and 24 adult (12 males, 12 females) rats were employed. The data are expressed as mean ± SEM.

ethanol predilection than adolescents. More important, the three-way interaction between Day of assessment, Mode of exposure and Sex ($F_{2,114} = 3.20$, $p \leq 0.05$; $\eta^2 p = 0.05$) was also significant. The subsequent planned comparisons indicated that females exhibited fairly similar ethanol predilection in the pre- and post-test, despite the mode of ethanol exposure during the binge phase. On the contrary, control males – but not those given binge or i.p. ethanol exposure – exhibited increased ethanol predilection vs. water in the in the post-test, compared to the pre-test.

The ANOVA for water intake (ml/100 g of body weight) revealed significant main effects of Sex and Age ($F_{1,114} = 12.70$, $p \leq 0.001$; $\eta^2 p = 0.10$ and $F_{1,114} = 6.89$, $p \leq 0.01$; $\eta^2 p = 0.06$, respectively). Females and adults drank more water than males and adolescents at the two-bottle choice ethanol intake tests conducted before and after binge exposure, and these effects were not affected by the group in which the rats were assigned, nor there were significant interactions between the factors. Water intake scores can be found in **Table 1**.

Behavioral Responsiveness After Exposure to Binge Ethanol (Exp. 1)

Behavioral responsiveness data in the LDB, NOR, and MSCF is presented in **Table 2**, as a function of age of first ethanol exposure and mode of ethanol exposure. The data is presented collapsed by sex (male, female). As detailed in this section, sex did not exert, for the most part, significant main effects nor was involved in significant interactions. Thus, to facilitate data presentation, data has been collapsed by sex in **Table 2**.

Light-Dark Box Test

Latency to enter into the black section of the LDB and time spent in the white section (**Table 2**, upper section) were significantly greater in adolescent than in adults ($F_{1,118} = 12.72$, $p < 0.001$; $\eta^2 p = 0.10$ and $F_{1,118} = 32.69$, $p < 0.001$; $\eta^2 p = 0.22$, respectively) yet not affected by the remaining factors, nor the ANOVA yielded significant interactions. Number of transfers (**Table 2**) was significantly lower in adolescents vs. adults, $F_{1,118} = 35.75$, $p < 0.001$; $\eta^2 p = 0.23$.

NOR Test

The analysis of activity scores during the habituation phase of the NOR protocol revealed significant main effects of Age ($F_{1,118} = 20.04$, $p < 0.001$; $\eta^2 p = 0.14$, lower activity in adult than in adolescents) and Mode of exposure ($F_{1,118} = 3.98$, $p < 0.05$; $\eta^2 p = 0.06$). As shown in **Figure 4**, ethanol exposure – either i.p. or binge drinking – exerted suppressive effects upon activity levels, which seemed specific for adolescents. Separate Mode of Exposure × Age ANOVAs conducted in total activity scores across the 10-min session confirmed this impression. The ANOVA for adults did not reveal significant main effects or

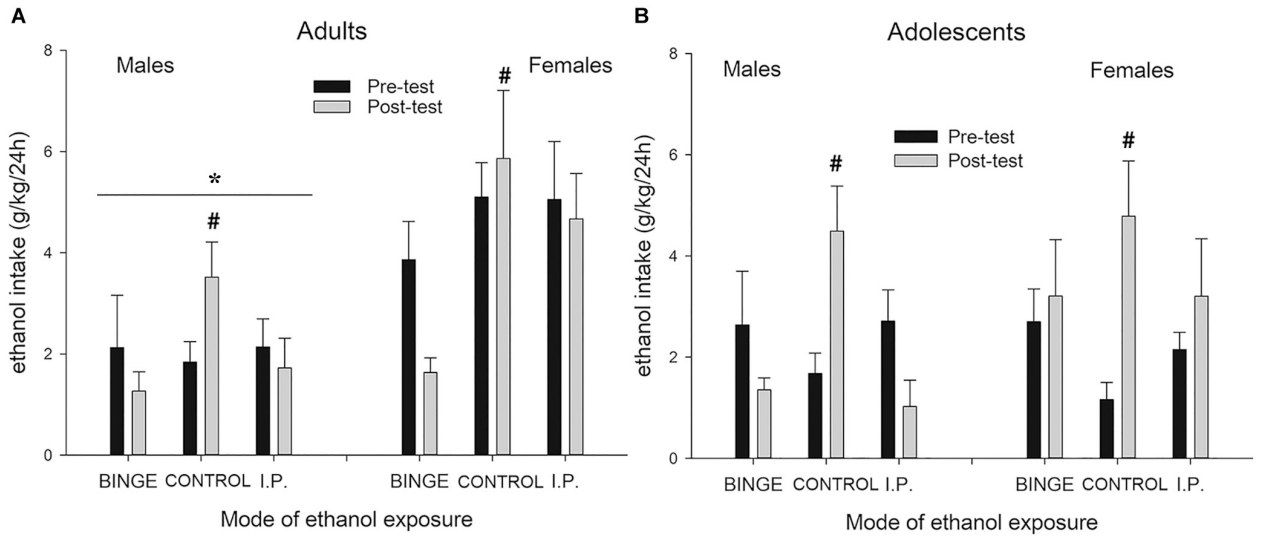

FIGURE 3 | Ethanol intake (g/kg) (**A** and **B** panels, respectively) during two-bottle, 24 h free-choice tests, in male and female Wistar rats as a function of group assignment (BINGE, CONTROL or IP). The rats were assessed on a two-bottle, 24 h free-choice, test on PD 30 or 70 (adolescent or adult groups, pre-test before any treatment) and on PD 56 or PD96 (i.e., post-test after termination of the binge-like exposure). During the BINGE/IP/CONTROL exposure phase the BINGE group underwent 10 sessions in which the rats were exposed to a bottle of 8% (first two sessions) or 10% ethanol (third and subsequent session) between 1900 and 2100 h. After each session the rats in the IP group were given an intraperitoneal administration of ethanol whose dose was matched for the level of ethanol ingestion exhibited by their same-sex counterparts of the binge group. CONTROL rats were undisturbed during the binge/ip exposure phase. The statistical analysis indicated that females drank more than males [an effect indicated by the asterisk (*) sign] and that, at the post-test, the control groups – either male or females, adolescent or adults – drank significantly more ethanol than rats that had been binging or had received i.p. administrations of ethanol. The latter effect is indicated by the hashtag (#) sign. The BINGE group employed 19 adolescent (9 males, 10 females) and 24 adult (12 males, 12 females) rats, the CONTROL group employed 20 adolescent (12 males, 8 females) and 24 adult (12 males, 12 females) rats, and the IP group employed 18 adolescent (8 males, 10 females) and 24 adult (12 males, 12 females) rats. The data are expressed as mean ± SEM.

TABLE 1 | Water (ml/100 g of body weight) ingested during the 24-h two-bottle choice tests.

	Adolescents			Adults		
	Binge	**Control**	**I.P.**	**Binge**	**Control**	**I.P.**
Females						
Pre-test	8.14 ± 0.97	8.60 ± 2.06	7.21 ± 1.25	12.08 ± 1.37	9.77 ± 1.22	9.63 ± 1.97
Post-test	8.41 ± 0.77	7.85 ± 0.97	9.63 ± 1.36	10.25 ± 0.90	10.11 ± 1.09	10.86 ± 1.48
Males						
Pre-test	4.19 ± 0.79	6.82 ± 1.62	6.56 ± 0.96	9.10 ± 1.19	6.07 ± 1.52	7.92 ± 1.50
Post-test	6.00 ± 0.86	6.06 ± 1.3	7.63 ± 2.03	8.23 ± 1.14	7.14 ± 1.25	7.55 ± 1.31

significant interactions. In contrast, the analysis for adolescents revealed significantly lower motor activity in ethanol-exposed rats – either via binge drinking or i.p. exposure – than in controls ($F_{2,52}$ = 4.06, $p < 0.05$; η^2p = 0.13). Sex did not exert a significant main effect nor was involved in significant interactions. Thus, to facilitate data visualization, data has been collapsed by sex in **Figure 4**.

During the first phase of the NOR protocol, the rats were introduced in the arena that had explored the day before and were exposed for 5 min to two identical objects (i.e., A and A'). The ANOVA on time spent in the vicinity of the objects at this phase did not reveal a significant main effect of "object," nor this factor interacted with Sex, Mode of Exposure or Age. This indicated that there was no innate preference for A or A' across the groups. Yet, the ANOVA and the subsequent tests indicated that total time spent in the vicinity of the objects (i.e., time spent close to A + time spent close to A') was significantly greater in adolescents, but not adult, rats exposed to i.p. ethanol than in controls (significant interaction between Mode of Exposure and Age, $F_{2,111}$ = 4.93, $p < 0.01$; η^2p = 0.08). Adolescents that underwent binge drinking also exhibited a trend (p = 0.07) toward greater overall object exploration than controls. These results are in **Table 2** (middle section).

During the 5 min NOR test the RM ANOVA (i.e., considering time spent in the vicinity of each object as a repeated measure) yielded a significant main effect of Object and a significant interaction between Object and Age ($F_{1,112}$ = 66.30, $p < 0.001$; η^2p = 0.37 and $F_{1,112}$ = 7.38, $p < 0.01$; η^2p = 0.06, respectively). As shown in **Table 1** and confirmed by the *post-hoc* tests, the rats spent significantly more time near the novel object B than

TABLE 2 | Behavioral responsiveness measured in each behavioral test.

			Adolescents			Adults		
			Binge	**Control**	**I.P.**	**Binge**	**Control**	**I.P.**
LDB	Latency to enter the black side (s)		14.53 ± 2.21*	17.10 ± 2.50*	12.10 ± 1.82*	7.67 ± 1.05	9.87 ± 1.34	11.29 ± 1.16*
	Transitions (freq.)		3.00 ± 0.43	2.05 ± 0.25	2.74 ± 0.35	21.29 ± 4.66*	23.71 ± 4.98*	16.33 ± 4.07*
	Time in white side (s)		34.42 ± 5.26*	31.80 ± 4.79*	41.26 ± 6.07*	14.17 ± 5.33	12.21 ± 4.53	9.21 ± 4.42
NOR PHASE 1	Time spent (s)	A object	13.47 ± 1.93	9.40 ± 1.22	15.59 ± 1.87	10.54 ± 0.75	11.42 ± 0.93	10.62 ± 1.07
		A' object	13.58 ± 1.35	9.35 ± 1.05	16.18 ± 2.18	11.62 ± 0.73	11.63 ± 0.78	12.21 ± 0.51
		A + A'	27.05 ± 3.05	18.75 ± 2.03#	31.76 ± 3.92	22.17 ± 1.24	23.05 ± 1.41	22.83 ± 1.33
NOR TEST	Time spent (s)	A	11.79 ± 1.18	8.58 ± 1.06	9.76 ± 1.57	8.17 ± 0.71	10.67 ± 0.76	10.71 ± 0.96
		B or Novel	19.05 ± 1.91*	12.74 ± 1.56*	15.35 ± 1.81*	10.96 ± 0.81	13.67 ± 0.87	13.62 ± 1.04
		A + B	30.84 ± 2.44#	21.31 ± 2.26	25.12 ± 2.99	19.12 ± 1.23	24.33 ± 1.32	24.33 ± 1.81
	Di		0.22 ± 0.05*	0.22 ± 0.07*	0.26 ± 0.07*	0.12 ± 0.07	0.12 ± 0.04	0.13 ± 0.03
MSCF	RAMP	Time spent	69.04 ± 9.63	65.18 ± 11.24	75.90 ± 17.21	82.48 ± 11.52	87.67 ± 11.81	103.53 ± 11.19
		Entries (f)	5.00 ± 0.54	4.50 ± 0.96	5.27 ± 1.07	8.21 ± 1.93	10.21 ± 1.53	11.04 ± 1.43
	BRIDGE	Time spent	12.79 ± 5.37	21.22 ± 9.06	13.48 ± 6.66	40.22 ± 11.07*	68.87 ± 16.81*	54.31 ± 11.06*
		Entries (f)	0.74 ± 0.28	1.20 ± 0.41	0.80 ± 0.24	2.21 ± 0.45	3.54 ± 0.72	3.29 ± 0.61
	CHA	Time spent	16.24 ± 8.44	47.15 ± 14.73	15.97 ± 10.29	90.30 ± 16.36	64.49 ± 16.52	83.53 ± 13.07
		Entries (f)	0.74 ± 0.31	1.30 ± 0.36	0.73 ± 0.36	3.75 ± 0.54	2.58 ± 0.58	3.87 ± 0.57
	SHELTER	Time spent	267.47 ± 35.92	148.37 ± 26.55&	329.80 ± 50.23	155.89 ± 17.81	165.95 ± 18.24	163.26 ± 19.60
		Entries (f)	9.63 ± 0.56	6.95 ± 1.03	10.20 ± 0.74	8.87 ± 0.75	11.00 ± 0.98	9.54 ± 0.87
	OF	Time spent	389.38 ± 24.95	405.12 ± 19.24	335.89 ± 39.11	353.28 ± 18.73	354.99 ± 20.24	376.08 ± 20.15
		Entries (f)	32.79 ± 1.40	29.85 ± 2.17	31.13 ± 3.9	34.21 ± 1.75	37.46 ± 2.09	36.96 ± 2.40
	PASS	Time spent	433.89 ± 17.14	425.02 ± 22.52	416.43 ± 21.36	423.77 ± 18.34	413.34 ± 20.39	374.92 ± 14.13
		Entries (f)	47.84 ± 1.89	42.30 ± 3.62	46.13 ± 4.00	52.42 ± 3.19	58.42 ± 3.24	58.96 ± 3.61

*Data gathered in the light-dark box (LDB) test, the novel object recognition (NOR) test and the multivariate squared concentric field (MSCF) test. Data are expressed as mean ± SEM, after collapsing by sex. The latter did not exert, for the most part, significant main effects nor was involved in significant interactions. Thus, to facilitate data presentation, data is collapsed by sex. Di, discrimination index, CHA, challenge area, OF, open-field, PASS, passages. * indicates a significant main effect of AGE (i.e., adolescent vs. adult), in a given variable. # indicates that total time spent in the vicinity of the objects was significantly greater in adolescent rats exposed to i.p. ethanol or to binge ethanol than in the other groups. & indicates that time spent in the SHELTER was significantly greater in adolescents exposed to i.p. or binge ethanol than in controls. Time spent in each section of the MSCF apparatus is expressed in seconds. Please refer to the text for a full account of the significant main effects and significant interactions found. All $p \leq 0.05$.*

near the familiar object A, an effect that was significantly greater in adolescents than in adults yet was not affected by the history of ethanol exposure (i.e., the interactions comprising Mode of exposure and Object were not significant, all $p > 0.05$). It is worth mentioning, however, that total time spent in the vicinity of the objects (i.e., time spent close to A + time spent close to B) was, in adolescents but not in adults, affected by Mode of exposure. Specifically, the interaction between Age and Mode of exposure achieved significance ($F_{2,112} = 7.49$, $p < 0.001$; $\eta^2 p = 0.12$) and the *post-hoc* tests revealed that adolescents exposed to binge drinking spent significantly more time at test exploring the objects – regardless their novelty or familiarity – than i.p. or control counterparts. Descriptive data (mean ± SEM) of time spent exploring the objects at the test can be found **Table 2**.

The ANOVA on discrimination (Di) scores revealed a significant main effect of Age ($F_{1,112} = 6.02$, $p < 0.05$; $\eta^2 p = 0.05$), with adolescents exhibiting greater Di scores than adults. None of the remaining factors nor the interactions between them achieved significance. Discrimination scores are presented in the middle section of **Table 2**.

MSCF Test

Time spent and frequency of entries in the different sections of the apparatus is shown in **Table 2**, lower section. The ANOVA of overall locomotor activity during the test – i.e., total frequency of transfers between compartments – revealed a significant main effect of age ($F_{1,114} = 17.70$, $p < 0.001$, $\eta^2 p = 0.13$; i.e., greater motor activity in adult than in adolescent rats) that did not interact with the other factors. This indicates that time spent in the different sections of the maze was not affected by ethanol-induced alterations in motor activity.

An important result was that shelter seeking was not affected by mode of ethanol exposure in those rats had been exposed to ethanol as adults, yet it was significantly greater in adolescents exposed to i.p. or binge ethanol than in controls (significant age × treatment interaction: $F_{2,114} = 4.95$, $p < 0.001$, $\eta^2 p = 0.08$, descriptive data shown in **Table 2**). More in detail, IP or BINGE adolescents exhibited a circa two-fold increase in time spent in the dark and enclosed SHELTER. Time spent in these sections was also greater in females than in males ($F_{1,114} = 9.98$, $p < 0.001$, $\eta^2 p = 0.08$).

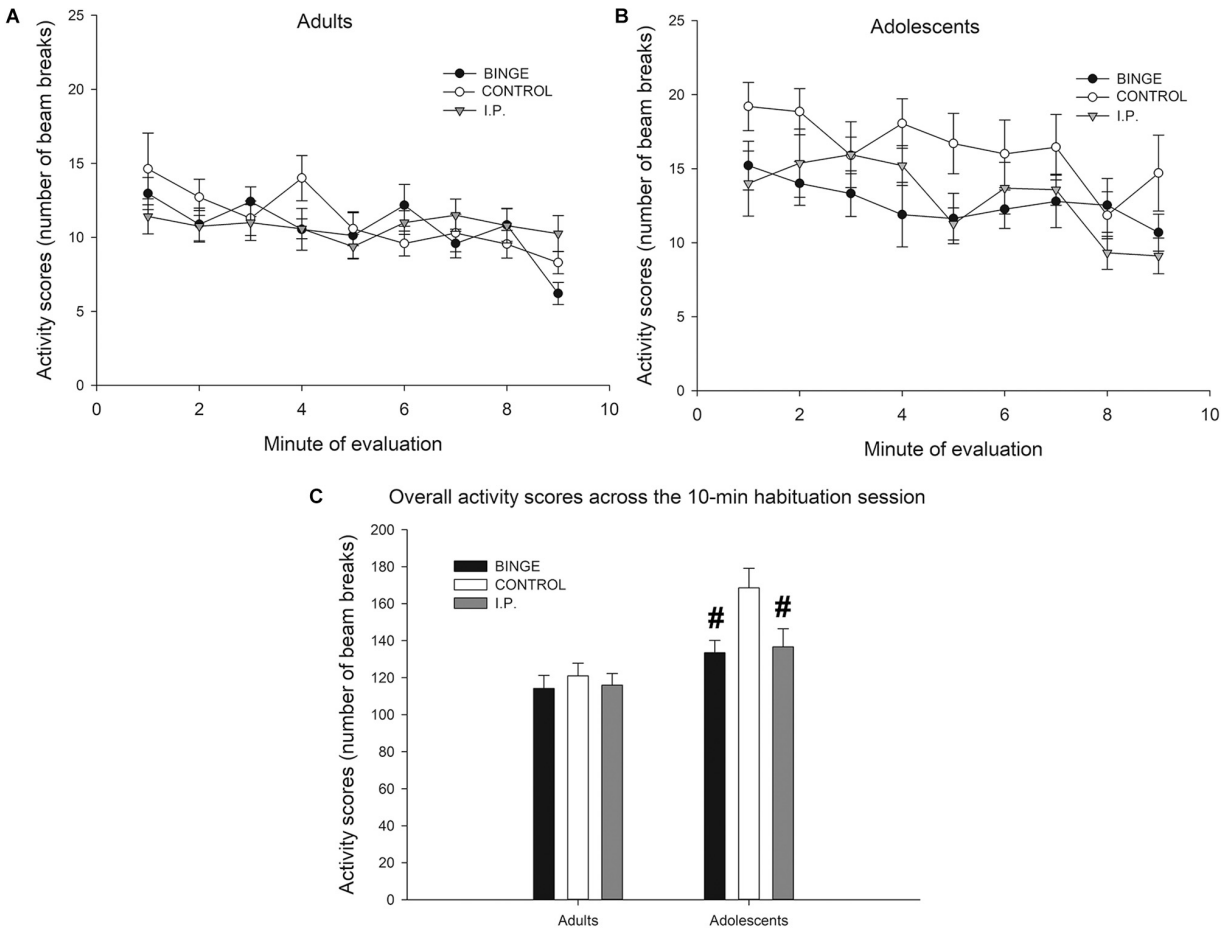

FIGURE 4 | (A,B) Activity scores (number of beam breaks) of Wistar rats in an open-field like test conducted at postnatal day 112, as a function of age of first ethanol exposure (adolescence, adulthood; panels **A** and **B**, respectively) and mode of ethanol exposure (BINGE, CONTROL or I.P.). The BINGE treatment consisted of 10 sessions in which the rats were exposed to a bottle of 8% (first two sessions) or 10% ethanol (third and subsequent session) between 1900 and 2100 h. After each session the rats in the IP group were given an intraperitoneal administration of ethanol whose dose was matched for the level of ethanol ingestion exhibited by their same-sex counterparts of the binge group. CONTROL rats were left undisturbed. Sex did not exert a significant main effect upon activity scores, nor was involved in significant interactions. Thus, to facilitate data visualization, data has been collapsed by sex. **(C)** Same data as in upper panel but expressed as total activity counts during the 10-min session. The statistical analysis indicated that the rats first exposed to ethanol as adolescents (either via self-administration or via i.p. administrations) exhibited significantly lower activity scores than same age controls. This effect is indicated in panel **C** via hashtag (#) signs. The BINGE group employed 19 adolescent (9 males, 10 females) and 24 adult (12 males, 12 females) rats, the CONTROL group employed 20 adolescent (12 males, 8 females) and 24 adult (12 males, 12 females) rats, and the IP group employed 18 adolescent (8 males, 10 females) and 24 adult (12 males, 12 females) rats. The data are expressed as mean ± SEM.

Time spent in the BRIDGE ($F_{1,114}$ = 16.99, $p < 0.001$, $\eta^2p = 0.13$) was significantly greater in adults than in adolescents, a pattern also found among female rats in terms of time spent in the RAMP or CHA areas (significant sex × age interaction, $F_{1,114} = 4.56$, $p < 0.05$, $\eta^2p = 0.04$ and $F_{1,114} = 7.06$, $p < 0.001$, $\eta^2p = 0.06$, respectively). Time spent in the OF was lower in IP females than in IP males, yet similar among male and females given binge or i.p. ethanol exposure (significant sex × treatment interaction, $F_{2,114} = 5.07$, $p < 0.01$, $\eta^2p = 0.08$).

Two-Bottle Choice Ethanol Intake Tests Conducted at Late Adulthood (Exp. 1)

The long-term effects of age of first ethanol exposure and the mode of exposure of such experience (i.e., 10 binge or i.p. ethanol exposures plus two 24 h choice tests between ethanol and water or – CONTROL group – only the two 24 h choice tests) were assessed in 24 h-long, two-bottle, ethanol intake tests. These tests took place at late adulthood, on PDs 120–139.

The ANOVA for g/kg ingested yielded significant main effect of Age of first exposure ($F_{1,115} = 6.90$, $p < 0.01$; $\eta^2p = 0.57$) and significant Sex × Day, and Age × Day interactions ($F_{8,920} = 3.06$, $p < 0.05$; $\eta^2p = 0.03$ and $F_{8,920} = 4.97$, $p < 0.001$; $\eta^2p = 0.04$, respectively). The ANOVA for percent ethanol predilection, in turn, revealed significant main effects of Age of first exposure and Day ($F_{1,115} = 4.73$, $p < 0.05$; $\eta^2p = 0.04$ and $F_{8,920} = 5.94$, $p < 0.001$; $\eta^2p = 0.05$, respectively) and a significant Age of first exposure × Day interaction ($F_{8,920} = 3.71$,

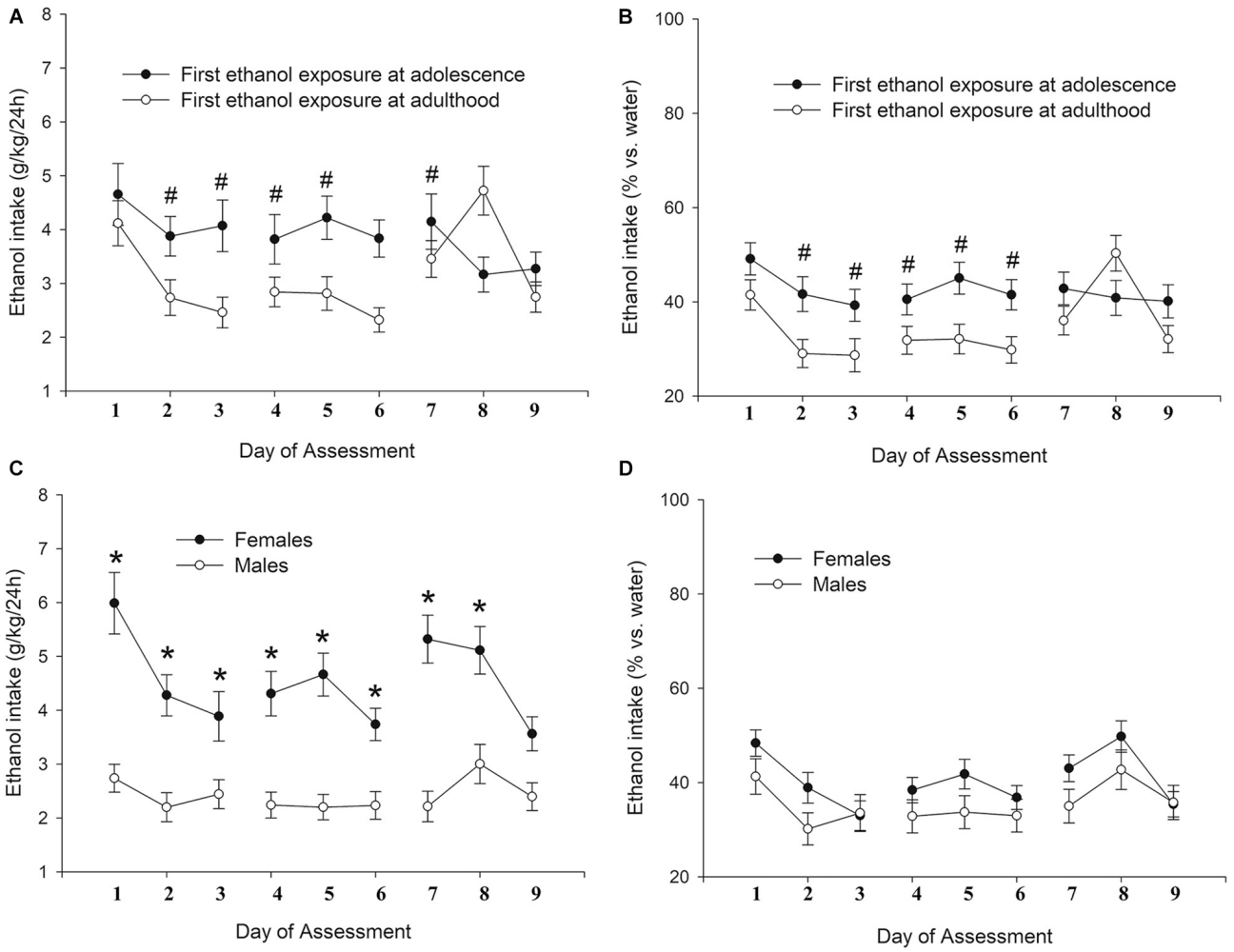

FIGURE 5 | Ethanol intake (g/kg and percent preference, panels **A** and **C**, and panels **B** and **D**; respectively) in Wistar rats as a function of sex (females, males, panels **C**,**D**) or age of first ethanol exposure (adolescence, adulthood; panels **A**,**B**) and day of assessment (9 intermittent two-bottle intake tests conducted at PDs 120–139). Data in panels **A** and **B** is present collapsed across sex, whereas data in panels **C** and **D** is presented collapsed across age of first ethanol exposure. The statistical analyses indicated that the rats that had been first exposed to ethanol at adolescence exhibited, when compared to those that had been first exposed as adults, significantly greater ethanol intake and ethanol percent predilection at sessions 2, 3, 4, 5, and 6. These significant differences are indicated by the hashtag (#) sign. The analyses also indicated that female rats drank more (g/kg) ethanol than male rats in sessions 1–8, which is indicated by the asterisk sign. There were 57 rats first exposed to ethanol at adolescence (29 males, 28 females), whereas 72 were first exposed to ethanol as adults (36 males, 36 females). The data are expressed as mean ± SEM.

$p < 0.001$; $\eta^2 p = 0.03$). The *post-hoc* tests indicated that the rats that had been exposed, and thus initiated to ethanol, to the BINGE/IP/CONTROL procedures at adolescence exhibited greater ethanol intake and ethanol percent predilection than those that had been exposed as adults, an effect that achieved significance at sessions 2, 3, 4, 5, and 6. Also, female rats drank more ethanol (g/kg) than male rats in sessions 1–8. **Figure 5** depicts absolute and percent ethanol intake as a function of age of first ethanol exposure, and day of assessment. The upper panels (A,B) present the data collapsed across sex, whereas the lower panels (C,D) present the data collapsed across age of first ethanol exposure.

Binge Drinking After Naloxone Administration (Exp. 2a), Two-Bottle Choice Ethanol Intake Tests Conducted Before and After Binge Drinking Exposure (Exp. 2a) and BELs Registered on Binge Session 3 (Exp. 2b)

Experiment 2a exposed adolescent rats to six sessions of the binge protocol. In sessions 1 and 2 they were given vehicle administration prior to the test, whereas in sessions 3–6 they were administered naloxone, 30-min prior to the 2-h access to ethanol. Two-bottle ethanol intake tests (length: 24 h) were conducted

48 h before and 48 h after the binge protocol. The ANOVA for g/kg ingested during the binge protocol revealed significant main effects of naloxone and a significant interaction between Naloxone and Session ($F_{1,18}$ = 37.03, $p < 0.001$; η^2p = 0.67 and $F_{5,90}$ = 8.86, $p < 0.001$; η^2p = 0.33, respectively). As shown in **Figure 6** and confirmed by the pair-wise comparisons, ethanol intake was similar across all rats in sessions 1 and 2 (i.e., when all rats were treated with vehicle), yet on sessions 3–6 the rats given naloxone drank significantly less than dose given vehicle. Naloxone administration was associated with a 2-fold reduction in ethanol intake. Ethanol ingestion during the baseline two-bottle choice test was similar in the rats that would be treated with naloxone or vehicle. In contrast, ethanol intake at the two-bottle choice test conducted after the binge exposure revealed significantly lower ethanol intake in rats treated with naloxone during the binge vs. those given vehicle (t_{16} = 2.75, $p < 0.05$). There was no drug administration immediately before these tests.

Experiment 2b exposed six male adolescent rats to a two-bottle choice between 8% ethanol, followed by two 2-h binge sessions in which they had access to 8% ethanol. At binge session 3 they drank 10% ethanol before being decapitated. Mean ethanol intake (g/kg) achieved during each measurement is depicted in **Figure 6**. The mean BEL registered at the end of binge session 3 was 60.82 ± 22.39 mg/dl. There was a positive and significant correlation between the BELs registered at the end of binge session 3 and the g/kg ingested by the rats during that session (r = 0.87, $p < 0.05$).

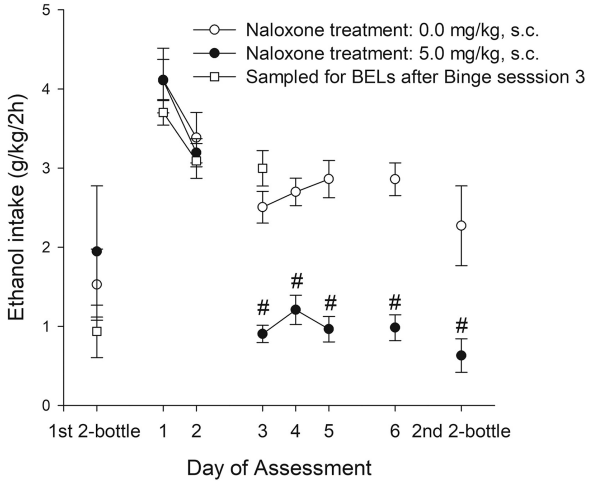

FIGURE 6 | Ethanol intake (g/kg) in male adolescent rats of Experiment 2a and 2b. Data for Experiment 2a is presented as a function of binge intake session (i.e., Day of Assessment 1–6) and naloxone treatment (0.0 or 5.0 mg/kg, subcutaneous, s.c.) applied 30 min before commencement of binge sessions 3–6. On each binge session the rats were exposed to a bottle of 8% (first two sessions) or 10% ethanol (third and subsequent sessions) between 1900 and 2100 h. The rats were assessed on a two-bottle, 24 h free-choice, test on PD 30 (pre-test before exposure to binge) and a post-test conducted 48 h after termination of the binge-like exposure. The statistical analyses indicated that, on binge sessions 3–6 and on the two-bottle choice test conducted after the binge exposure, the rats given 5.0 mg/kg naloxone drank significantly less than dose given vehicle. These significant differences are indicated by the hashtag (#) sign. Twenty rats were employed (10 administered naloxone, 10 administered vehicle). The figure also depicts (i.e., white squares) mean ethanol intake (g/kg) achieved in six male adolescent rats (Exp. 2b) that underwent the first two-bottle choice test and three 2-h binge sessions. These rats were decapitated at termination of binge session 3 and blood samples were obtained and processed for blood ethanol levels. The data are expressed as mean ± SEM.

DISCUSSION

A main result was the dramatic difference in binge-like ethanol drinking between adolescent and adult rats. Up to a 3-fold difference was observed between these groups, an effect most noticeable in males than in females and in the initial than in the latter binge sessions. The results agree with epidemiological studies indicating that adolescents drink less often than adults, yet when they do they ingest significantly greater quantities (Windle and Zucker, 2010). Specifically, it has been shown that adolescents drink more than twice as much than adults per drinking occasion (Substance Abuse and Mental Health Services Administration, 2006). A nationally representative study reported, the ingestion of 38.8 and 80.1 g of alcohol per consumption episode in individuals aged ≥65 or 14–24 years respectively (Servicio Nacional para la Prevención y Rehabilitación del Consumo de Drogas y Alcohol, 2017).

Pre-clinical studies also suggest that, under different conditions and settings, adolescents drink more than adults, albeit the evidence is much less abundant in rats than in mice (Doremus et al., 2005). For instance, C57BL/6J adolescent mice given DID-like ethanol access (2 h per night) consumed significantly more than their adult counterparts, an effect that persisted 3 weeks later, when both groups of mice were adults (Moore et al., 2010). Yet, the results are far from being conclusive. In the latter study DBA/2J mice showed no adolescent vs. adult difference, neither in the initial DID phase nor in the second phase conducted 3 weeks later. Another study (Younis et al., 2019) applied the DID procedure in adolescent or adult C57BL/6J mice and found, unlike our work, similar drinking of 20% ethanol across 9 (i.e., between 6 and 8 g/kg/4 h).

DID-like or scheduled access to ethanol has been much less employed in rats, albeit some success has been achieved when using lines selectively bred to show innate preference for ethanol (Bell et al., 2014). Nowak et al. (1999) and McKinzie et al. (1998) reported that adult female or male, alcohol-preferring (P), rats consumed ∼2 g/kg in 2 h-long sessions conducted during the dark phase. Sardinian alcohol-preferring rats, on the other hand, drank ≤1.0 g/kg ethanol when the 2 h drinking sessions occurred immediately after lights off (Colombo et al., 2017). These levels of ethanol consumption, achieved by adult rats derived from lines selected for high alcohol consumption, are generally lower than those found in the genetically heterogeneous adolescents of the present study, which drank 2.5–3.0 g/kg/2 h in the first week of limited, binge-like, access to ethanol and 2.0–2.5 g/kg/2 h in the subsequent weeks. Interestingly, the latter levels are similar to those reported by Bell et al. (2011) in adolescent P rats exposed to a limited access binge-like procedure.

Also interesting is that the greater DID-like drinking reported in adolescent vs. adult rats or mice seems particularly noticeable in social situations. Logue et al. (2014) reported very little adolescent vs. adult mice differences in a short (45 min, housed one animal per cage) session of access to 5% ethanol, yet the adolescents drank significantly more if tested with a companion. Our study, conducted in rats, did not systematically vary social conditions, yet the animals were tested in their homecage and separated from the partner via a lid that prevented touching but not smelling or hearing. It is possible that these conditions favored greater ethanol intake in the youth vs. the adults.

The greater binge drinking of the adolescents was particularly noticeable during the early sessions, yet as testing progressed they merged their level of intake with that shown by adults. More in detail, binge-like consumption decreased over time in the adolescents, an effect particularly noticeable in male rats. These results seem to clash with those from studies [for review and references, see Carnicella et al. (2014)] suggesting an escalation of ethanol consumption in rats when employing intermittent alcohol exposure protocols, commonly referred to as intermittent access to ethanol in 2-bottle choice [IA2BC, e.g., Maier et al. (2019)]. The latter literature, however, has focused on adult rats, whereas the result we are discussing was exhibited by adolescent subjects. Interestingly, the patterns displayed by these adolescents are reminiscent of those reported by Bell et al. (2011) and by Truxell et al. (2007). The latter authors exposed adolescent or adult rats to the consumption-off-the floor paradigm, in 3-daily sessions spread across a 5-day period, and observed that ethanol ingestion significantly decreased over time in so-called juveniles (i.e., tested at PDs 25–28) or in adolescents tested at PDs 30–34, yet ethanol ingestion remained stable in young adult rats tested at PDs 60–64. It is thus possible that the pattern displayed by the adolescent rats, in the DID-like section of the present study, may reflect a normative decrease in ethanol acceptance, as the animals transition from adolescence to adulthood. It is worth noting that, during the course of the protocol, we employed increasing concentrations of alcohol (from 8 to 10%), yet these were still lower than those employed in other DID-like procedures [e.g., 20% (Pavon et al., 2016)]. Perhaps different ethanol self-administrations patterns would have been observed had we employed higher ethanol concentrations, or had we kept the ethanol concentration stable across sessions.

Another aim was to analyze effects of the binge-like ethanol exposure upon exploratory and anxiety responses, and cognitive performance. It has been shown that ethanol administration (3.0–5.0 g/kg, i.p.) throughout adolescence impairs conditioned discrimination learning (Pascual et al., 2007), reversal spatial learning in the Morris water maze (Coleman et al., 2011), and short-term recognition memory in the NOR test and in an odor-habituation test (Montesinos et al., 2015). Some studies suggest that rats or mice given similar treatments at adulthood are spared from these effects. A study (White et al., 2000) gave rats 5.0 g/kg i.p. ethanol every other day over 20 days, beginning at PD 30 or 70. At a subsequent test in a radial arm maze, the rats treated with ethanol at adolescence – but not those treated at adulthood – exhibited working memory impairments. Short- and long-term spatial memory, however, was unaffected, as well as anxiety responses in an EPM. Similar lack of alterations in anxiety response after adolescent or adult i.p. binge exposure were observed in Wistar rats (Fabio et al., 2014).

In the present study the effects of binge ethanol exposure upon cognitive or exploratory responses were observed only when ethanol exposure occurred at adolescence. Ethanol exposure at adolescence, either i.p. or binge, significantly reduced the exploration of the open field-like chamber in which the NOR training took place. Reduced propensity to explore novel environments suggests an anxiety-like profile, and has been found after stress (Berridge and Dunn, 1986; Pautassi et al., 2012). The possibility that ethanol exposure induced an anxiety-prone phenotype in adolescents, but not in adults, is consistent with the finding that shelter-seeking in the MSCF test was significantly increased in adolescents exposed to i.p. or binge ethanol, but not altered in control adolescents or in adults.

Repeated ethanol administration (2.0–4.0 g/kg, once daily for a total of 7–8 administrations) has been shown to impair cognitive performance in the NOR test in rats (Marszalek-Grabska et al., 2018) and in mice (Wolstenholme et al., 2017). Moreover, Marco et al. (2017) reported performance deficits in the NOR test, in male and female Wistar rats that self-administered ethanol (20% in drinking water) four times a week during PDs 28–52. Unlike these studies, in the present work novelty object recognition was preserved after ethanol exposure, with adolescents and adults preferring the novel over the known object. Discrimination scores, however, were significantly higher in adolescents than in adults, a result probably obeying to the greater levels of novelty preference normatively exhibited by adolescent, when compared to adults (Stansfield and Kirstein, 2006; Walker et al., 2017). Binge- or experimenter-administered, ethanol-induced, alterations could probably have been observed if we had employed the spatial variant of the NOR test. The latter, but not the NOR, test is sensitive to hippocampal alterations (Jablonski et al., 2013), and we (Fernandez et al., 2019) and others (Hunt and Barnet, 2016) have shown that ethanol treatments akin to those of the present study yield alterations in the adolescent – but not in the adult – hippocampus of the rat.

Our ethanol-exposed adolescents, however, did show alterations during the NOR protocol, in terms of the overall level of exploratory activity. Specifically, during the familiarization phase time spent in the vicinity of the objects was greater in IP or BINGE adolescents than in CONTROLS. This effect was also observed during the NOR test, albeit in the BINGE group only. This result indicates that adolescent ethanol exposure affected exploratory patterns in the NOR test, although this effect did not translate to alterations in cognitive performance (i.e., novelty object recognition was preserved after ethanol exposure).

As expected, the binge exhibited by the adolescents was blocked by acute pre-treatment with naloxone. The blockade of the opioid system reduces, in humans, the enhancement in mood ratings found the after drinking ethanol (Davidson et al., 1999) and, in rats, blocks ethanol-induced behavioral stimulation, ethanol-induced conditioned place preference (Pautassi et al., 2011) and ethanol drinking (Shoemaker et al., 2002). An interesting result of Experiment 2a was that naloxone had a

lingering effect, reducing ethanol drinking vs. vehicle-treated controls at the 2nd two-bottle choice test, long after its clearance. An important limitation was that we did not assess naloxone effects upon baseline water ingestion, which detracts from the specificity of the effect reported in Experiment 2a. Moreover, the binge pattern reported was observed after a mild (50% of the water usually consumed by the rats) yet significant water restriction. We did not include a group of rats that had access to the binge sessions without water restriction, so we can not dissect the influence of this procedural factor.

In Exp. 1, greater binge drinking at adolescence did not enhance 24 h 2-bottle choice drinking, neither when tested immediately after the binge sessions nor at adulthood. Age of first exposure to ethanol, however, did affect level of intake during the tests conducted at late adulthood, with rats that had been initially exposed to ethanol at adolescents drinking significantly more than those that had similar exposure during early adulthood. This permissive effect of adolescent ethanol exposure upon adult ethanol drinking was similar in IP or BINGE groups, indicating that the effect was not dependent on the intensity of such exposure; and emerged even after the brief experience with the drug of CONTROL subjects, which were exposed to ethanol during the two 24-h choice tests. In other words, adolescent binge ethanol exposure did not enhance later, free-choice, drinking at adulthood to a greater length than i.p exposure. Instead, it seems that any kind of adolescent exposure to ethanol during adolescence, even the brief level experienced by those in the CONTROL condition, is sufficient to enhance the proclivity to ingest and prefer the drug, when compared to subjects given similar ethanol exposure but during adulthood. These results agree with clinical and pre-clinical work that argue in favor of the "early debut" effect (Vera et al., 2019; Younis et al., 2019), yet differ from studies that suggest that this effect is more likely to be expressed by those that experienced levels of intoxication consistent with a drunkenness or binge episode (Kuntsche et al., 2013; Fabio et al., 2014).

It could be argued that none of the adolescents in our study achieved levels of intoxication compatible with the definition of binge, as the mean BEL registered at the termination of binge session 3 in Exp. 2b (i.e., 60.82 ± 22.39 mg/dl) was shy from the 80 mg/dl threshold commonly used to define binge drinking (e.g., Hosova and Spear, 2017). Yet it should be noted that we measured BELs 120 min after the commencement of the binge, and others have suggested that schedules of restricted drinking induce most of the intake during the initial 30–60 min (McKinzie et al., 1998; Nowak et al., 1999). BELs also had the confound of being assessed after ethanol exposure (i.e., the two-bottle choice tests the binge sessions 1 and 2).

Another relevant methodological detail is that we equated the age of testing for both group of rats (i.e., those exposed to alcohol as adolescents or as adults) and tested them at the same age at adulthood. A caveat of this procedure is that the delay between the last two-bottle choice session at the first exposure phase and the first two-bottle test at adulthood is different between the two age groups (i.e., substantially shorter for those exposed to alcohol for the first time as adults). This confound was also present during the behavioral assessments at PDs 110–113 (i.e., LDB, MSCF, and NOR tests). It is possible that lingering withdrawal effects were still present at that moment and, thus, affected anxiety-like responses differentially in adolescent and adults.

A dissociation was observed in regards with the effect of sex on ethanol intake. During the two-bottle intake tests ethanol intake was significantly greater in females than in males, regardless age; yet ethanol intake during the binge drinking sessions was affected by sex (i.e., significantly greater in females than in males) in adult rats only. It is possible this dissociation obeys to female adolescent rats exhibiting a functional ceiling effect during the binge sessions, that prevented them from exhibiting their (relative to males) high-drinking phenotype (Li et al., 2019). It should be noted that the greater consumption of the females on the 1st evaluation of ethanol intake may have had carry-over effects, affecting the drinking levels observed afterward. Greater ethanol intake in female than in male rats is a consistent finding in pre-clinical research, yet the emergence of the phenomenon is affected by testing conditions. For instance, a study that employed the two-bottle choice test reported (Penasco et al., 2015) greater ethanol intake in female than in male Wistar rats, yet only after exposure to a week of alcohol cessation combined with restraint stress.

Another limitation of the study is that, in Experiment 1, the significantly greater ethanol consumption observed in adolescents vs. young adults occurred in rats that were water deprived and only had access a to single ethanol bottle. It certainly conceivable that these results obey, at least partially, to age-related differences in thirst (Kenney and Chiu, 2001). This possibility cannot be discarded. It is important to remark, however, that baseline water consumption during the two-bottle choice intake test conducted before binge drinking exposure indicated that water intake per gram of body weight was higher in adult than in adolescent rats. This effect of greater water intake in adult than in adolescent rats was also observed during the *post-test* intake session, which took place after binge exposure.

Effect sizes of the most relevant significant main effects or significant interactions reported were relatively variable. Most of the significant effects reported for the analysis of ethanol intake scores were medium size (i.e., η^2p = 0.06–0.13), albeit the reduced *post-test* ethanol intake observed in BINGE and IP groups (relative to controls) was associated with a big effect size. A big effect size was also found for naloxone's consequences upon ethanol intake (η^2p = 0.33). Effect sizes for the significant main effects or significant interactions reported for the variables measured at the LDB, MSCF, or NOR tests were also highly variable, with most falling within the medium size effect range.

Despite the limitations, the study cements the notion that binge-like ethanol drinking is substantially greater in adolescent that in adults, an effect that can be normalized by blockade of opioid transmission. Ethanol exposure at adolescence, but not at adulthood, was associated with altered response to novel stimuli and greater subsequent ethanol intake at late adulthood. The results support the notion that preventing alcohol access to adolescents should reduce the likelihood of problematic alcohol use and alcohol-related consequences.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC (CICUAL).

AUTHOR CONTRIBUTIONS

ASa, ASu, LR-L, and RP run the intake tests at adolescence and adulthood. RP, CC, IM, ASu, ASa, and LR-L had the original scientific idea, designed the study and analyzed the data. ML run the behavioral assays and analyzed that section of the data. LR-L run Experiment 2b and processed the blood samples. ASu, RP, and LR-L wrote the initial draft of the manuscript. All authors participated in the subsequent writing of the manuscript and gave approval to the final form.

FUNDING

Funding was provided by grants PICT 2015-0325 and SECYT Consolidar 2018 to RP and by a post-doctoral and international exchange fellowships awarded by ANPyCT and Universidad de Granada to ASu and LR-L, respectively.

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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.

frontiers in Psychiatry

ORIGINAL RESEARCH
published: 09 June 2020
doi: 10.3389/fpsyt.2020.00535

Response Inhibition and Binge Drinking During Transition to University: An fMRI Study

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Addictive Disorders, a section of the journal Frontiers in Psychiatry

Received: *04 February 2020*
Accepted: *26 May 2020*
Published: *09 June 2020*

Citation:
Suárez-Suárez S, Doallo S, Pérez-García JM, Corral M, Rodríguez Holguín S and Cadaveira F (2020) Response Inhibition and Binge Drinking During Transition to University: An fMRI Study. Front. Psychiatry 11:535. doi: 10.3389/fpsyt.2020.00535

Background: Binge Drinking (BD), a highly prevalent drinking pattern among youth, has been linked with anomalies in inhibitory control. However, it is still not well characterized whether the neural mechanisms involved in this process are compromised in binge drinkers (BDs). Furthermore, recent findings suggest that exerting inhibitory control to alcohol-related stimuli requires an increased effort in BDs, relative to controls, but the brain regions subserving these effects have also been scarcely investigated. Here we explored the impact of BD on the pattern of neural activity mediating response inhibition and its modulation by the motivational salience of stimuli (alcohol-related content).

Methods: Sixty-seven (36 females) first-year university students, classified as BDs (n = 32) or controls (n = 35), underwent fMRI as they performed an alcohol-cued Go/NoGo task in which pictures of alcoholic or non-alcoholic beverages were presented as Go or NoGo stimuli.

Results: During successful inhibition trials, BDs relative to controls showed greater activity in the bilateral inferior frontal gyrus (IFG), extending to the anterior insula, a brain region usually involved in response inhibition tasks, despite the lack of behavioral differences between groups. Moreover, BDs displayed increased activity in this region restricted to the right hemisphere when inhibiting a prepotent response to alcohol-related stimuli.

Conclusions: The increased neural activity in the IFG/insula during response inhibition in BDs, in the absence of behavioral impairments, could reflect a compensatory mechanism. The findings suggest that response inhibition-related activity in the right IFG/insula is modulated by the motivational salience of stimuli and highlight the role of this brain region in suppressing responses to substance-associated cues.

Keywords: binge drinking, response inhibition, Go/NoGo, fMRI, alcohol-related stimuli

INTRODUCTION

Alcohol is by far the most used drug among youth in Western countries, as informed in epidemiological reports by the ESPAD (*European School Survey Project on Alcohol and Other Drugs*) (1) and the SAMSHA (*Substance Abuse and Mental Health Services Administration*) (2). The actual consumption rate of this substance entails significant health and economic costs (3, 4) and is one of the main causes of death among young people and adolescents (4). In this regard, multiple studies have indicated that the age of onset of drinking may be a determining factor in the development of future alcohol use disorders (AUD), illicit drug dependence and different problem drinking patterns (5–9). For example, Hingson et al. (8) informed that an early drinking onset significantly increases the probability to engage in binge drinking (BD). This pattern of consumption, characterized by the intake of large amounts of alcohol in a short period of time (leading to a blood alcohol concentration of at least 0.08 g/dl) [National Institute on Alcohol Abuse and Alcoholism (NIAAA), (10)], has been linked to neural and neuropsychological anomalies (11–13) and it could be considered as an initial step for developing alcohol use disorders (7, 14, 15). Moreover, evidence from animal (16) and human (17) studies about the vulnerability of the adolescent brain to the neurotoxic effects of alcohol highlights the impact of alcohol consumption on brain development. Of particular concern is the upsurge in alcohol consumption that takes place once the legally allowed age is exceeded (i.e. 18–21 years in most countries) but brain maturation is still under development (18, 19). In this regard, previous studies have demonstrated that brain regions known to support cognitive control, such as the prefrontal cortex, mature late (20, 21) and are particularly vulnerable to the neurotoxic effects of alcohol consumption (22, 23). University students have been specifically identified as a population of interest, mainly due to the escalation in alcohol drinking and increased rates of BD during transition to university (24, 25), placing them in a vulnerable position to develop future AUD (15).

Neuroscientific models of addictive behaviors have proposed that impairments of two related processes—response inhibition and salience attribution—may underlie the development of substance use disorders (26–29). In line with dual-process models, evidence of an imbalance between impaired response inhibition and an increased impact of the motivational properties of drug-related stimuli has been reported in alcohol-dependent patients [for a review, see (30)]; however, less is known about this potential imbalance in young binge drinkers (BDs) [for a theoretical framework, see (31)].

Response inhibition, usually defined as the ability to withhold or suppress a prepotent response (32), is considered a key mechanism to adjust behavior to meet environmental demands. Different meta-analyses have revealed the involvement of a predominantly right-lateralized fronto-parietal network, including the inferior parietal lobule (IPL), inferior frontal gyrus (IFG), middle frontal gyrus (MFG) and anterior insula, in successful inhibition of responses (33–38), and have underlined the importance of the right inferior frontal cortex (36, 37, 39).

An extensive body of work in alcohol dependence has shown alterations in inhibitory control at behavioral and neural levels [for a review, see (40); for a meta-analysis, see (41)]. Studies centered on young BDs have also provided evidence for the hazardous effects of this pattern of consumption on inhibitory control processes [for a review, see (42)], although its impact on the neural network subserving response inhibition is still not well characterized. In this regard, neuroimaging studies have reported an increased neural activity in BDs compared to controls during successful response inhibition trials [(43); see also (44)], as well as the recruitment of different brain regions during failed inhibitions (45), even in the absence of behavioral differences between the groups.

Regarding the enhanced salience attribution to drug-related stimuli in alcohol-dependent patients, a recent meta-analysis has revealed increased neural activity in brain regions implicated in incentive salience, reward processing and habit circuitry (e.g. dorsal striatum, prefrontal areas, anterior cingulate cortex and insula) (46). This study has also indicated the presence of differences between heavy and light drinkers in the activity of parietal and temporal regions (46). Regarding non-clinical BDs, fMRI studies that assessed alcohol cue reactivity and implicit positive associations towards alcohol cues reported similar results, showing greater neural activity in BDs in comparison with light drinkers in several incentive salience- and reward-related areas including, but not limited to, the anterior cingulate cortex, insula and dorsal striatum (47, 48). Furthermore, greater neural activity to alcohol-related pictures in some of these regions predicted increases in drinking and more alcohol- related problems in a group of college students who transitioned to heavy drinking during a year follow-up period (49).

In line with the findings mentioned above and the principles of dual-process models, one could expect to find greater response inhibition impairments to alcohol-associated stimuli in both individuals with AUD and BDs. Studies with alcohol-dependent patients offer behavioral and neuroimaging evidence that supports this hypothesis. A recent systematic review indicates that patients with AUD tend to show increased recruitment of the inhibitory control neural network (comprising dorsolateral and ventrolateral prefrontal cortex) and the salience network (anterior cingulate, insula and IPL) during alcohol-related processing while showing decreased engagement of relevant brain networks during non-drug-related processing (29). This potential imbalance has been, however, scarcely investigated in BDs, with the few published studies reporting, at a behavioral level, both the presence (50) and absence (51–55) of differences in the percentage of false alarms to alcohol-related stimuli, and with, to our knowledge, only one neuroimaging study trying to disentangle the subjacent neural mechanisms of these effects (56). In this work, Ames et al. (56), using a Go/NoGo task that required the inhibition of a prepotent response to alcohol images (NoGo stimuli), reported an increased neural activity in BDs, compared to controls, in regions involved in cognitive control (i.e. dorsolateral prefrontal cortex, anterior cingulate and anterior insula) during successful inhibition trials, in the absence of behavioral differences in the proportion of inhibitory errors. However, this study only

included alcohol images as NoGo stimuli, and it is thus not possible to determine if the observed neural response pattern was specifically related to successful inhibition of response to alcohol-related stimuli or to a more general response inhibition process.

Here, we performed event-related functional magnetic resonance imaging (fMRI) in first-year university students during an alcohol-cued Go/NoGo task with a twofold aim: (1) to investigate the association between BD and potential anomalies in inhibitory control, and (2) to examine whether response inhibition processes are affected by the motivational salience of stimuli (i.e. alcohol- or non-alcohol-related content). Based on previous findings (43), we hypothesized, first, that BDs, compared to controls, would show an increased activation during successful inhibition trials (independently of the type of stimulus) in brain areas commonly identified as involved in response inhibition, such as the IFG, anterior insula, MFG or the IPL (33, 35), in the absence of behavioral differences between groups. Second, in line with previous studies (56) we expected the pattern of neural activity mediating response inhibition to be modulated by the stimulus motivational value, reflected in increased engagement, in BDs relative to controls, of the response inhibition neural network when withholding a response to alcohol-related stimuli. At a behavioral level, based on previous findings (51–56) we did not expect to find any significant differences between groups in the proportion of inhibitory errors.

MATERIALS AND METHODS

Participants

Eighty-five first-year university students (18-19 years old) were selected to participate in the neuroimaging assessment within the framework of a broader research on consequences of BD among university students (for ERPs results, see 54). Initially, 2,998 first-year students from the University of Santiago de Compostela (Spain) completed a classroom questionnaire assessing alcohol and other substance consumption, as well as sociodemographic information. This questionnaire included the adapted version of the Alcohol Use Disorders Identification Test (AUDIT) (57, 58), the short version of the Nicotine Dependence Syndrome Scale (NDSS-S) (59, 60) and the Cannabis Abuse Screening Test (CAST) (61, 62). In order to identify the most suitable participants among the initial 2,998 questionnaires, the following preselection criteria were applied to the classroom questionnaire: i) provision of contact information (phone number and/or email); ii) 18–19 years old; and iii) non-consumption of illegal drugs (except cannabis). From the initial 2,998 questionnaires, a total of 516 subjects were identified to meet these criteria and showed interest in participating in the study. These participants completed a semi-structured interview in which quantity and frequency of alcohol use over the past 180 days were assessed *via* the Timeline Follow-Back calendar (TLFB) (63). Additionally, those subjects who reported cannabis consumption at some time throughout their lives during the classroom questionnaire completed the Cannabis TLFB to assess their cannabis consumption over the past 90 days. Participants were also interviewed about personal and family history of psychopathological disorders and completed the Spanish version of the Symptom Checklist-90-Revised (SCL-90-R) (64) to ensure they met inclusion/exclusion criteria. Exclusionary criteria included the following: chronic medical conditions that could affect neurocognitive functioning (diabetes, hypothyroidism, liver diseases, etc.), history of neurological disorders or brain injury, personal history of DSM-IV-TR Axis I and/or II diagnosed disorders, a score above 90th percentile in the Global Severity Index (GSI) or in two or more symptoms dimensions of the SCL-90-R, family history of major psychopathological disorders in first-degree relatives (clinically diagnosed by a professional), family history of alcoholism or substance use disorders (at least two first-degree relatives or three or more first- or second-degree relatives), AUDIT scores > 20, use of psychoactive medications, use of illegal drugs (except occasional consumption of cannabis) in the last 6 months, non-corrected sensory deficits and MRI contraindications. All participants gave written consent and received monetary compensation for their participation.

Volunteers were classified as binge drinkers (BDs) if they reported one BD episode at least once a month for the last six months, or as controls (CN) if they did not reach the alcohol consumption threshold for being considered BDs. Binge episodes were defined as the consumption of ≥ 50 g (female) or ≥ 70 g (male) of alcohol in one drinking occasion (i.e. an equivalent measure of the 4/5 standard drinks criteria reported in the NIAAA's definition of BD) (10). Participants were instructed to abstain from consuming alcohol 24 h prior to the scan session.

Of the 85 subjects who met the inclusion criteria and completed the neuroimaging assessment, five participants were excluded from the analysis due to technical problems during image acquisition, seven participants were excluded due to excessive head movement during scanning (more than 3 mm/degrees of movement in any of the six directions), five were excluded for outlier behavioral data (more than 3 SD above or below the group mean) and, lastly, one was excluded due to the presence of an artefact in the functional images. Hence, the final sample included 67 right-handed participants, with 32 BDs (20 females) and 35 CN (16 females) (see **Table 1** for complete demographic and alcohol use data).

Behavioral Task

During fMRI, participants completed a Go/NoGo task with pictures of alcoholic or non-alcoholic beverages as stimuli (see **Figure 1** for

TABLE 1 | Demographic and substance use characteristics of the final sample (mean ± SD).

	Controls	BDs
n (females)	35 (16)	32 (20)
Age	18.08 ± 0.28	18.22 ± 0.42
Caucasian (%)	100	100
Age of drinking onset***	16.29 ± 1.04	15.22 ± 1.24
Total AUDIT score ***	1.94 ± 2.52	10.28 ± 4.06
Number of BD episodes, past 6 months ***	0.91 ± 1.75	22.91 ± 12.26
Average # drinks per drinking occasion ***	1.96 ± 1.53	6.78 ± 1.98

****p* ≤.001,

AUDIT, Alcohol Use Disorders Identification Test; BD episode: consumption of ≥ 5 (female) or ≥ 7 (male) Spanish standard drinks (10 g of alcohol) in 1 occasion.

illustration). The picture set was designed to include drinks representative of Spanish consumption habits, comprising active pictures that display beverages being served, opened or consumed, following similar criteria to the Amsterdam Beverage Picture Set (ABPS) (65). Forty-eight pictures displaying different alcoholic beverages (beer, wine, and spirits) were used as alcohol-related stimuli, whereas 48 pictures displaying water, juice, dairy and soft drinks were used as non-alcoholic stimuli. All pictures had the same background and were scanned at a similar resolution and image size.

Stimuli were presented using the software Presentation (version 16.3, Neurobehavioral Systems Inc., Albany, CA; http://www.neurobs.com/) and were delivered through MRI-compatible video goggles (VisualSystem, NordicNeuroLab, Bergen, Norway), with a resolution of 800 x 600 pixels. The task included two blocks of 168 images each (126 Go trials and 42 NoGo trials). Each picture stimulus was presented on a light gray background for 300 ms at the center of the screen followed by a long and variable inter-stimulus interval (ISI) lasting 2–12 s, during which only a fixation cross was displayed. The durations for the ISIs were drawn from a logarithmic distribution that was skewed toward the shorter intervals (50% 2–4 s, 33% 4–8 s, 17% 8–12 s) (66). At the beginning of each block, participants were instructed to respond to one type of stimulus (Alcohol or Non-Alcohol), pressing a button with their right index finger on an MRI-compatible response grip (NordicNeuroLab, Bergen, Norway), as quickly as possible without making errors (Go trials), and to withhold their response to the other stimulus type (NoGo trials). Trial type (Go vs. NoGo) was randomized within each block and the order of blocks (Go Alcohol vs. Go Non-Alcohol) was counterbalanced across subjects.

Participants received a practice session of the task before they entered the scanner. Once in the scanner, and prior to each block, participants were informed, through a 3-s instructions screen, about the type of stimulus to which they must respond (i.e. Go Alcohol or Go Non-Alcohol trials).

Behavioral Analysis

Reaction times (RTs) and the percentage of correct responses to Go stimuli, as well as the percentage of false alarms (FA) (i.e. response to NoGo stimuli), were submitted to 2x2x2 mixed-model analyses of variance (ANOVAs), with stimulus type (Alcohol, Non-Alcohol) as the within-subjects factor and group and gender as the between-subjects factors. Post-hoc comparisons were performed using the Bonferroni adjustment for multiple comparisons. All analyses were done with SPSS (version 21).

fMRI Data Acquisition

Functional images were collected with a 3T Achieva Philips body scanner (Philips Medical Systems, Best, NL) equipped with a 32-channel SENSE head coil (located at the University Hospital Complex of Santiago de Compostela) using a T2*-weighted echo-planar imaging sequence with the following acquisition parameters: TR/TE = 3000/30 ms, flip angle = 87°, FOV = 230 × 230 mm, voxel size = 3 mm^3, 45 axial slices. The task was conducted in one run consisting of about 700 volumes (~35 min). High-resolution anatomical T1-weighted images were also acquired using a 3D turbo field-echo sequence with the following parameters: TR/TE = 7.7/3.4 ms, flip angle = 8°, FOV = 240 mm, voxel size = 0.8 mm^3, 200 transverse slices, acquisition time = 7 min.

Image Processing and Analysis

Imaging data were processed and analyzed using Statistical Parametric Mapping (SPM8; http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) implemented in Matlab (version 2015b, The Mathworks, Inc., Natick, MA). First, functional and anatomical images were reoriented to the anterior commissure. Then, functional images were corrected for slice timing and realigned and unwarped to correct for movement artefacts. The anatomical T1 images were coregistered to the realigned mean functional image, then images were transformed into standard MNI space using segmentation-based normalization parameters. The resulting functional images were spatially smoothed using a 7-mm FWHM Gaussian kernel. Blood oxygen level-dependent (BOLD) responses to each condition were modeled using an event-related design convolved with the canonical hemodynamic response function (HRF) to create regressors of interest (Go

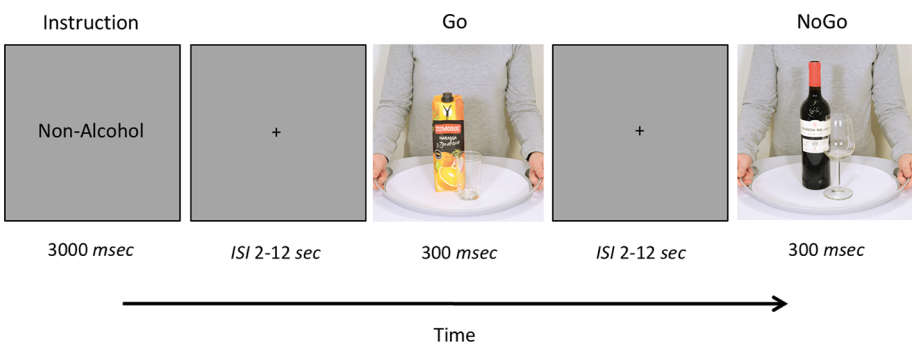

FIGURE 1 | Schematic representation of the Go/NoGo task. Participants completed a Go/NoGo task with pictures of beverages (with or without alcoholic content) as stimuli. The task consisted in two blocks of 168 trials (25% NoGo trials). Before each block, participants were instructed to respond to one type of stimuli (Alcohol or Non-Alcohol) and to withhold their response to the other stimulus type.

Alcohol, Go Non-Alcohol, NoGo Alcohol, NoGo Non-Alcohol). Instruction screens and response errors (i.e. failures to respond on Go trials or FA on NoGo trials) were modeled as effects of no interest. Additionally, movement parameters from the realignment step were included in the design matrix as regressors of no interest.

Individual t-contrasts were generated for each participant and then entered into a second-level random-effects analysis. Gender was included as a covariate in the analysis. We examined, first, the main effect for the response inhibition contrast (NoGo > Go) across all participants. The statistical threshold was set at $p < .05$ family-wise error (FWE) corrected for multiple comparisons at the voxel level across the whole brain.

Secondly, two-sample t-tests were defined to determine the between-group effects (i.e. BD > CN, BD < CN). Given our *a priori* hypotheses regarding differences in brain activity related to response inhibition (and its modulation by motivational salience), region of interest (ROI) analyses were conducted based on areas defined from a meta-analysis of Go/NoGo tasks involving complex stimulus identification (33) as follows: right IPL, right IFG, left IFG, right MFG, and right superior frontal gyrus (SFG) (see **Supplementary Table 1** for a list of ROIs coordinates). Contrasts were initially thresholded at $p < .005$ uncorrected and a cluster extent of 10 voxels; small volume correction (SVC) for multiple comparisons was then applied with a FWE-corrected threshold of $p < .05$ at cluster level within 10 mm-spheres centered on the reported coordinates after their transformation from Talairach into MNI space. To investigate the predicted higher BOLD activity in brain regions involved in response inhibition in BDs relative to CN, we examined the contrast NoGo > Go. Next, we examined modulation of NoGo vs. Go trial activation by the alcohol- vs. non-alcohol-related stimulus content. Our prediction of greater engagement in BDs (vs. CN) in areas mediating response inhibition when stimuli convey motivational salience was tested by a two-sample t-test comparing NoGo Alcohol > Go Non-Alcohol trials. To ensure that potential group differences in this contrast were directly linked to inhibiting responses to alcohol-related stimuli, and not simply due to general differences in inhibitory control processes, activation in NoGo Non-Alcohol > Go Alcohol trials was also explored. Finally, to test whether any observed activation differences between groups were due to overall differential reactivity to alcohol cues, we explored the contrast Alcohol > Non-Alcohol.

Additional Pearson's correlation analyses were performed between parameter estimates extracted from each ROI showing significant between-group differences and: i) the age at drinking onset; ii) the number of BD episodes in the last 6 months, as a measure of the intensity of the BD pattern.

RESULTS

Behavioral Performance

There were no significant differences between groups (BDs vs. CN), neither in the response to Go stimuli (i.e. percentage of hits or RTs) nor in the number of commission errors (i.e. percentage of FA) to NoGo stimuli. A significant main effect of stimulus type for the percentage of hits [$\mathbf{F}_{(1, 63)} = 28.288$, $p < .001$] revealed greater accuracy for alcohol-related than for non-alcohol-related stimuli irrespective of the participant's consumption pattern. There was also a significant gender by group interaction for the percentage of correct responses to Go stimuli [$\mathbf{F}_{(1, 63)} = 7.59$, $p = .008$], which was explained by higher accuracy in males of the BD group (95.57 ± .96) relative to the CN group (92.88 ± .76) ($p = .032$), with no significant group differences in females (93.49 ± .74 vs. 95.36 ± .83; $p = .098$). Behavioral data are summarized in **Table 2**.

fMRI Results

Whole-brain analysis for the whole sample revealed significant BOLD activations during successful inhibition (NoGo > Go) in different areas of the right hemisphere including the precentral gyrus, IPL, IFG, MFG and SFG (see **Table 3**, **Figure 2**). These regions have been identified to be involved in Go/NoGo tasks in different meta-analysis (33–35). These results validate the ability of our task to tap into neural mechanisms related to response inhibition.

ROI analyses revealed significant differences between groups in BOLD response during successful response inhibition (NoGo > Go). Specifically, BDs showed greater activity, in comparison with CN, in the bilateral BA47 (IFG extending to the anterior insula) during NoGo relative to Go trials (**Table 4**, **Figure 3**). Furthermore, the NoGo Alcohol > Go Non-Alcohol contrast revealed a significant increased activation in this region (BA 47), restricted to the right hemisphere, in BDs relative to the CN group, when inhibiting a prepotent response to alcohol-related stimuli (**Table 4**, **Figure 3**). However, activations in the NoGo Non-Alcohol > Go Alcohol and Alcohol > Non-Alcohol contrasts did not reach statistical significance. This pattern of results suggests the modulation of the right IFG/insula activity to be specifically associated with suppressing responses to alcohol-related stimuli. The ROI analysis did not show any significant regions of increased activity in CN compared with BDs.

TABLE 2 | Behavioral data for Control and BD groups (mean ± SD).

Behavioral Performance	Controls		Binge Drinkers	
	Alcohol	Non-Alcohol	Alcohol	Non-Alcohol
Reaction Time (Go trials, ms)	632 ± 107	642 ± 100	632 ± 92	616 ± 86
% Correct responses (Go trials)	96.98 ± 3.26	91.04 ± 5.88	96.53 ± 4.84	92.01 ± 6.11
% False alarms (NoGo trials)	6.39 ± 5.80	6.94 ± 6.69	7.07 ± 6.36	7.66 ± 6.27

TABLE 3 | Regions activated at whole-brain analysis in the contrast NoGo > Go for the whole sample.

Region (BA)	Side	x(mm)	y(mm)	z(mm)	k	*t*
Precentral/Postcentral Gyrus (4/3)	Right	40	-14	56	571	7.05
Precentral/Postcentral Gyrus (6)	Right	60	-10	38	108	6.74
Superior/Inferior Parietal Lobule (7)	Right	32	-54	52	237	6.59
Inferior Frontal Gyrus (47)	Right	30	28	-2	49	6.20
Middle Frontal Gyrus (9)	Right	42	20	26	45	5.73
Superior Temporal Gyrus (22)	Right	56	-38	4	19	5.55
Superior/Middle Frontal Gyrus (9)	Right	32	46	34	20	5.51
Superior Occipital Cortex/Precuneus (7)	Right	26	-84	28	33	5.47

BA, Brodmann Area.
All results are significant at p <.05 whole-brain voxel-level FWE corrected and cluster size (k) ≥ 10.

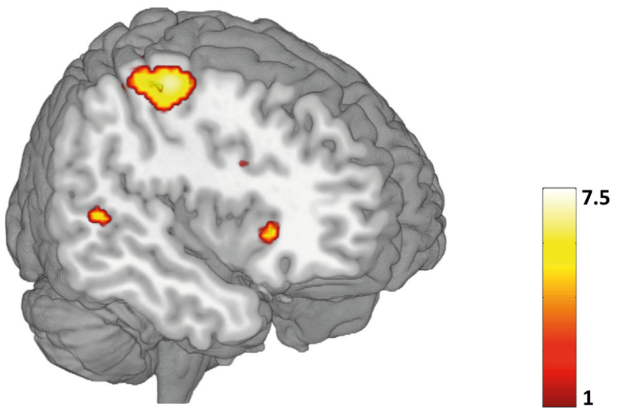

FIGURE 2 | Whole-brain task activation during correct inhibition (NoGo > Go) for the whole sample (p <.05, FWE corrected). Color bar represents t-values. A list of all significant activations for this contrast can be found in **Table 3**.

Correlation analysis performed in the BD group did not yield significant relationships (all p >.05) with any of the variables explored (i.e. age of onset of alcohol consumption, number of BD episodes in the last 6 months).

DISCUSSION

The main objective of this study was to investigate the link between BD and inhibitory control differences, with special consideration to the relationship between response inhibition and alcohol-related processing. We first analyzed the association between BD and behavioral performance in an alcohol-cued Go/NoGo task. We then explored the task-related neural correlates for both BDs and controls. Finally, we considered if there were differences in neural activity in BDs, relative to controls, when exerting inhibitory control to alcohol-related cues.

Consistent with most of the previous studies using Go/NoGo tasks in BDs (43, 67–72), we did not find behavioral differences related to BD in the examined response inhibition indices (i.e. FA) (irrespective of stimulus content). Furthermore, when alcohol cues were analyzed separately from non-alcohol cues, no significant differences in performance between BDs and CN were found. This finding is in line with a recent ERPs study from our group (54) and with previous studies reporting no differences in the number of FA (51–53, 55, 56), although some others have found an alcohol-cue-specific impairment of response inhibition (50).

In line with our hypothesis, the present neuroimaging results revealed an increased BOLD response in the bilateral IFG extending to the anterior insula (BA 47), in BDs compared to controls during successful inhibition trials, a region usually involved in response inhibition tasks (39, 73–75). As proposed by Aron and Poldrack (73), the inferior frontal cortex (IFC) modulates the interaction between the pre-supplementary motor area and the subthalamic nucleus (STN) within the neural network of response inhibition. Specifically, IFC sends excitatory impulses to the STN *via* the "hyperdirect" pathway and the STN sends excitatory output to the globus pallidus, which results in thalamus inhibition (76, 77). Looking at the characteristics of this neural circuitry, the increased activity in the IFG in the BD group could be interpreted as a greater recruitment of neural resources to successfully inhibit a response. Therefore, the differences observed in this region could be interpreted as part of a compensatory mechanism to attenuate the impact of abnormal brain activity on performance, in line with previous fMRI studies showing that BD is associated with increased neural activation, in the absence of behavioral impairments, during successful inhibition (43, 56).

TABLE 4 | Regions showing significant group differences (BD > CN) in BOLD response to successful inhibition.

Contrast	Region (BA)	Side	*P (FWE)**	x(mm)	y(mm)	z(mm)	k	*t*
NoGo > Go	IFG/Insula (47)	Right	0.031	34	34	-6	74	3.64
	IFG/Insula (47)	Left	0.018	-30	16	-2	114	3.30
NoGo Alcohol > Go Non-Alcohol	IFG/Insula (47)	Right	0.031	30	20	-6	72	3.57

ROI analyses were based on the coordinates informed in the meta-analysis by Criaud and Boulinguez (33).
**Small volume correction (FWE, p <.05).*

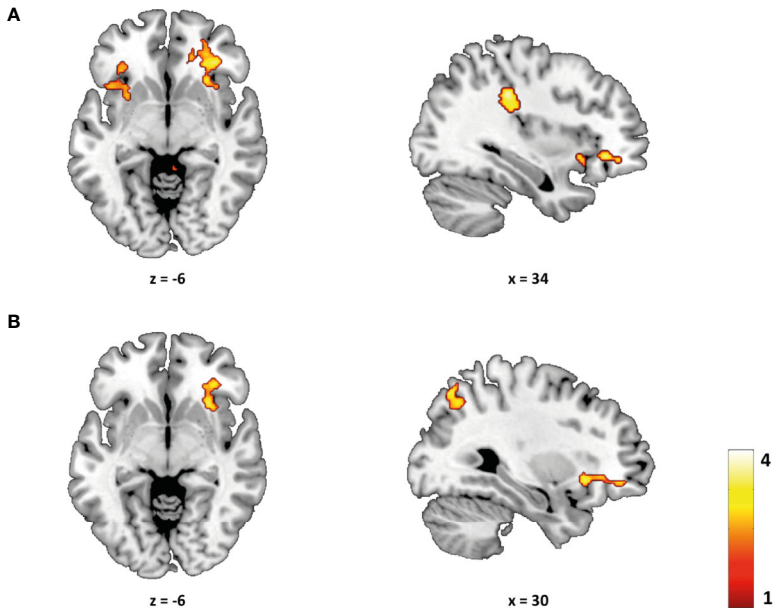

FIGURE 3 | Group differences (BD > CN) during successful inhibition. **(A)** Compared to controls, BDs showed a greater BOLD activity in the NoGo vs. Go contrast in the bilateral inferior frontal gyrus, extending to the anterior insula (BA 47) ($p < .05$ FWE-small volume corrected). **(B)** BDs, in comparison with controls, showed a greater BOLD activity in the NoGo Alcohol vs. Go Non-Alcohol contrast in the right inferior frontal gyrus/anterior insula (BA 47) ($p < .05$ FWE-small volume corrected). T-maps are thresholded at $p < .005$ (uncorrected) and $k \geq 150$ for display purposes only.

It is worth noting that while BD tends to be associated with increased neural response, AUD has been associated with reduced response in fronto-parietal regions (e.g., IFG, MFG, IPL) (78, 79). Further exploring this relationship, Worhunsky and colleages (80) have reported two different activity patterns associated with the escalation of maximum number of drinks consumed in a single episode (MaxDrinks). First, escalating drinkers showed a hyper-engagement of fronto-parietal control mechanisms during successful relative to unsuccessful inhibition trials compared to constant (low) drinkers. On the other hand, when the group of escalating drinkers was divided according to their MaxDrinks scores, a greater MaxDrinks was associated with reduced engagement of the fronto-parietal network. These findings thus suggest a transition, in terms of neural activation, from an initial consumption stage (related to hyperactivation) to a more problematic drinking (related to reduced engagement of neural resources).

Consistent with our second hypothesis and the findings reported by Ames et al. (56), we observed a greater activity in BDs (vs. controls) during response inhibition to alcohol-related stimuli. Specifically, BDs showed greater activity in the right IFG/insula (BA 47) when inhibiting a response to alcohol stimuli (NoGo Alcohol > Go Non-Alcohol). However, no significant differences were observed when participants were asked to inhibit their response to non-alcohol stimuli (NoGo Non-Alcohol > Go Alcohol). These findings suggest that response inhibition-related activity in the right IFG/insula seems to be modulated by the motivational salience of stimuli and highlight the role of this region in suppressing responses to substance-related cues. Also, these results are in agreement with previous studies, in the general population, showing that response inhibition could be modulated by the motivational content of stimuli (81–85). In particular, some of these works using fMRI to characterize the neural basis of this modulation reported increased neural activity of the IFG/insula in trials where a response to reward-related stimuli had to be inhibited (82, 85). It should be taking into account that the IFG and the anterior insula, besides being considered key regions for response inhibition, have been also proposed to be part of the salience network (82, 86, 87) and, therefore, they may be involved in adjusting cognitive control to motivational demands of the context (86). In this regard, a meta-analysis has suggested that the right anterior insula would play an important role in detection of behaviorally relevant salient events, whereas the right IFG would be more involved in exerting inhibitory control (36). Therefore, it is possible that the higher activity observed in BDs in the right BA 47 is pointing to the presence of differences associated with the alcohol consumption pattern in the interaction between cognitive control and salience detection processes.

The current research extends prior results about BD-related anomalies in inhibitory control mechanisms and provides new evidence about increased IFG/insula activity, a key region for inhibitory control, during response inhibition to alcohol-related stimuli. Some limitations of the present study should be noted. First, the lack of assessment before participants engaged in BD prevents us from establishing potential pre-existing differences between groups that may explain the observed results, as indicated by previous investigations (43, 88, 89). A future follow-up will

allow us to deepen the relationship between BD pattern and neural anomalies in frontal regions and to explore if these early neural differences may subtend the transition from recreational to pathological consumption patterns. Second, in contrast with how Go/NoGo tasks are typically designed to be performed in behavioral experiments, the characteristics of the hemodynamic response led us to employ a relatively slow and unpredictable stimulus presentation which could reduce the prepotent tendency to respond. Therefore, this type of approach may be less sensitive for assessing behavioral differences and it should be taken into account when explaining the lack of differences between the groups. Third, similarly to previous studies [e.g. (56, 90)], our task involves a much more demanding cognitive context than classic Go/NoGo tasks, with attention, stimulus categorization and response selection processes being highly intertwined, so research findings should be interpreted with this limitation in mind. One final limitation should be noted: neither individual preferences nor potential differences in familiarity of the employed images were addressed in the present study.

In summary, our results revealed that young BDs showed increased frontal activity, relative to controls, during successful inhibition trials in an alcohol-cued Go/NoGo task, despite a lack of behavioral differences between groups. These findings provide new evidence about the role of the IFG, extending to the anterior insula, as an important region to explore neural differences associated with BD, as well as suggest a specific involvement of this region in withholding a prepotent response to stimuli with alcohol-related content. At a more clinical level, our study provides subclinical information in a healthy population, without evidence of behavioral problems, which highlights the risk of this form of consumption. In addition, this investigation emphasizes the relevance of assessing the cognitive processes of interest using specific substance-related cues to better understand the relationship between those processes and the alcohol consumption pattern, and provides useful information to contribute to the development of future prevention strategies as suggested by studies focused on inhibitory control training and cognitive bias modification (see 91 for an insightful review of neuroscience findings and treatment programs).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethics Committee of the Universidade de Santiago de Compostela. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FC, SD, SR, and MC designed the study. SS-S and JP-G participated in the data collection. MC was responsible for sample selection. SD and SR designed the experimental task. SS-S analyzed the data. FC, SD, and SS-S interpreted the data. SS-S wrote the article. All authors contributed to the article and approved the submitted version.

FUNDING

This investigation was supported by grants from the Spanish Ministerio de Sanidad, Servicios Sociales e Igualdad, Plan Nacional sobre Drogas (PNSD 2015/034), Ministerio de Economía y Competitividad (PSI2015-70525-P) co-funded for European Regional Development Fund and Xunta de Galicia (GRC ED431C 2017/06). SS-S was supported by a predoctoral fellowship from the Spanish Ministerio de Economía y Competitividad (BES-2016-076298). JP-G was supported by the FPU program from the Spanish Ministerio de Educación, Cultura y Deporte (FPU16/01573).

ACKNOWLEDGMENTS

We thank to the personnel of the Radiology Service of the Clinical University Hospital of Santiago de Compostela (CHUS) for technical assistance in MRI data acquisition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2020.00535/full#supplementary-material

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 17 June 2020
doi: 10.3389/fnbeh.2020.00105

ELSA 2014 Cohort: Risk Factors Associated With Heavy Episodic Drinking Trajectories in Argentinean College Students

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *10 March 2020*
Accepted: *27 May 2020*
Published: *17 June 2020*

Citation:
Vera BV, Pilatti A and Pautassi RM (2020) ELSA 2014 Cohort: Risk Factors Associated With Heavy Episodic Drinking Trajectories in Argentinean College Students. Front. Behav. Neurosci. 14:105. doi: 10.3389/fnbeh.2020.00105

Heavy episodic drinking (HED) is highly prevalent in college students. In Argentina, there is a notable lack of longitudinal studies examining drinking trajectories. The present study identified HED trajectories in Argentinean college students during the first 3 years of college (seven waves) and examined the association between risk factors for alcohol use and HED trajectories. The sample was composed of 1,240 college students [63.1% women, aged 18–25 years (M = 19.1 ± 1.7)] who completed at least three waves (the first data collection and ≥2 follow-ups). For 3 years, participants completed seven surveys that measured HED frequency, age of drinking onset, drunkenness occurrence, trait impulsivity, family history of alcohol abuse, stressful life events, and perceived peer's drinking. Latent Class Growth Analysis (LCGA) and Multinomial Logistic Regression (MLR) were used to identify the pattern and number of HED trajectories and to explore which risk factors better distinguished between the trajectories, respectively. Six HED trajectories were identified: *Heavy Stable Frequency, Moderate Stable Frequency, Moderate Decreasing Frequency, Stable Infrequent, Decreasing Infrequent*, and *No-HED*. Younger age of drinking onset, alcohol intoxication, greater perception of peer drinking frequency and higher levels of impulsivity (i.e., sensation seeking, lack of premeditation, and positive urgency) increased the probability of belonging to the trajectories with more frequent HED. These trajectories partially coincide with those identified in studies from other cultures. Unlike previous studies, we did not find a trajectory with increasing/ascending HED frequency. This may be related to contextual/cultural variables unique to Argentina, like differences in the age when the peak in alcohol consumption is reached or the legal minimum age to buy alcoholic beverages in this country, and the idiosyncratic elements that characterize college life in Argentina. This work represents a step forward in the identification of risk factors differentiating between different HED trajectories, and help understand changes in alcohol use during college, in an understudied population.

Keywords: heavy episodic drinking, trajectories, risk factors, college students, Argentina

INTRODUCTION

Substance use is more frequent among emerging adults than in any other age group (Sedronar, 2017; Schulenberg et al., 2018). The transition from high school to college is a critical period for increments in alcohol use and alcohol-related negative consequences (Derefinko et al., 2016; Skidmore et al., 2016). Some evidence (Patrick and Terry-McElrath, 2017; Krieger et al., 2018; Schulenberg et al., 2018) suggests that alcohol use, and particularly heavy episodic drinking (HED), is more prevalent among college students than in their non-college peers.

HED is usually defined as the intake of ≥56/70 grams (women/men) of pure alcohol in a single, usually short, drinking occasion (Courtney and Polich, 2009). HED, which is highly prevalent (≈45–50%) among Argentinean emerging adults (Pilatti et al., 2014, 2017; Bravo et al., 2019) is associated with a wide variety of immediate and long-term negative consequences, such as academic problems, fainting, risky sexual behaviors, physical and sexual assaults, violent behavior, risk-taking and traffic accidents (Hingson, 2010; White and Hingson, 2013; Ferreira et al., 2014; Pilatti et al., 2016; Bravo et al., 2019). HED is also associated with an increased likelihood of developing alcohol (Kim et al., 2016; Cservenka and Brumback, 2017) or other substance use disorders (Dawson et al., 2010), and other mental health problems (Rehm, 2011). HED has also been shown to induce alterations in brain areas involved in drug reward, such as nucleus accumbens (Sousa et al., 2020).

Trajectory studies seek to explain behavior, including alcohol-related behavior, by accumulating data over time (Del Boca et al., 2004; Maggs and Schulenberg, 2004; Goudriaan et al., 2007). Most of the studies that examined alcohol use trajectories of college students found four types of trajectories (Greenbaum et al., 2005; Jackson et al., 2008; Sher et al., 2011; Derefinko et al., 2016): two stable (low/non-use or high use) and two dynamic (low/non-use that gradually increases over time, and other characterized by a high use that gradually decreases over time) trajectories. Some studies included a class of moderate alcohol use that remains stable over time (Ashenhurst et al., 2015; Derefinko et al., 2016) or a trajectory of low use punctuated by occasions of heavy drinking in specific periods, such as holidays (Greenbaum et al., 2005).

Successful interventions to reduce or delay alcohol drinking, or reduce the occurrence of HED and its negative consequences, should take advantage of identifying risk factors associated with these trajectories. Students engaged in heavy drinking trajectories exhibit, when compared to those of the other trajectories, higher trait impulsivity (Del Boca et al., 2004; Greenbaum et al., 2005; Jackson et al., 2008; Adams et al., 2013; Derefinko et al., 2016). Impulsivity is a multidimensional construct defined as the predisposition towards rapid and unplanned reactions, without considering the possible consequences (Moeller et al., 2001). Higher positive urgency, sensation seeking and lack of premeditation (i.e., facets of impulsivity, as suggested by the five-dimension UPPS-P impulsivity model; Lynam et al., 2007) are significant predictors of riskier drinking trajectories (Adams et al., 2013; Derefinko et al., 2016). Recent preclinical studies, in turn, suggest that the level of activation of cortico-mesencephalic tracts may predict greater vulnerability to the effects of binge drinking exposure on subsequent compulsive drinking (Siciliano et al., 2019).

Lower age of drinking onset and having experienced drunkenness (Casswell et al., 2002; Warner et al., 2007) are also significant predictors of riskier drinking trajectories. Strikingly, the odds of endorsing a heavy/risk drinking trajectory were significantly decreased with each additional year that drinking onset was delayed, and increased when participants reported they felt intoxicated during their first drinking episode (Warner et al., 2007). Peer factors, such as associating with peers who drink (Borsari and Carey, 2003), have been also related to engaging in heavy drinking trajectories (Chassin et al., 2002; Danielsson et al., 2010; Lee et al., 2012). Last but not least, a family history (FH+) of alcohol use disorders (Warner et al., 2007) and experiencing more stressful live events (Hanna et al., 2014) have been also regarded as genetic and environmental predictors, respectively, of riskier alcohol use trajectories.

The vast majority of studies on trajectories of alcohol use have been conducted with college students from the U.S. (Goudriaan et al., 2007; Sher et al., 2011) or Europe (Johnsson et al., 2008; Ståhlbrandt et al., 2010). In Argentina, the South American country where the present study was conducted, there is a notable lack of longitudinal epidemiological studies. Also, there are significant differences between the samples of college students employed in the previous studies and the Argentinean college students sampled in the present study. In fact, in Argentina, social organizations, such as fraternities or sororities, are non-existent and, different from the U.S. and some European countries, students cannot live on campus. Both features, availability of social organizations for students (Maggs et al., 2011; White and Hingson, 2013) and living on campus (Lorant et al., 2013) have been associated with heavier alcohol use. Additionally, in the U.S. the minimum legal age to buy alcohol is 21, whereas the minimum legal age is 18 in Argentina. Thus, Argentinean college students have legal access to alcohol during most of their college years. Noteworthy, in Argentina, different from the U.S. and most European countries, most college careers last between 5 and 6 years. Additionally, many careers require conducting final research to obtain a bachelor's degree which, in turn, adds one more year. These variations could impact the prevalence and type of drinking trajectories.

The present study aimed to identify HED trajectories in college students since college entry and during the first 3 years of college (seven waves). In addition to identifying HED trajectories, we examined the relations between HED trajectories and risk factors linked with the development or maintenance of alcohol use, including trait impulsivity, age of drinking onset, alcohol intoxication, family history of alcohol abuse, stressful life events and perceived peer's drinking. The study provides novel information, useful to understand changes in alcohol use during the college years of this understudied population, and, ultimately,

should help in prevention or intervention efforts to reduce heavy alcohol use and its associated burden.

MATERIALS AND METHODS

Participants

Participants were assessed seven times across 3 years: three times during the freshman year, twice during their sophomore year and twice during the third year of college. Four, out of the seven measures, were taken during the first semester of the academic year. The remaining three measures were taken during the second semester of the academic year. A total of 4,497 (57.8% women) college students completed the first assessment. However, only 1,977 could be contacted for the follow-ups (i.e., gave contact information). The sample analyzed in the present study (n = 1,240, 63.1% of women) comprises those who completed, at least, the first data collection, and ≥2 follow-ups. None of these measurements had to be necessarily consecutive. These participants had, at the commencement of the assessments, between 18 and 25 years old (M = 19.14, SD = 1.7). Demographic details can be found in **Table 1**. This sample of participants (n = 1,240) exhibited significant differences with those who dropped out of the study (n = 3,257). Specifically, those who dropped out from the study reported, compared to those who kept participating, significantly greater frequency of drinking (3.3 ± 2.9 vs. 2.9 ± 2.6 days, respectively; t = 4.738, $p \leq 0.001$), significantly greater grams of alcohol consumed per drinking occasion (119.5 ± 98.7 vs. 112.3 ± 93.3, respectively; t = 2.272, $p \leq 0.05$) and significantly greater alcohol-related negative consequences (8.7 ± 7.4 vs. 7.9 ± 6.8, respectively; t = 3.438, $p \leq 0.05$). Moreover, those who dropped out were, at the beginning of the assessments, significantly younger (t = 2.09, $p \leq 0.05$) than those who kept on participating (19.03 ± 1.6 vs. 19.14 ± 1.7 years, respectively), and men were more likely to be drop-outs group than women (χ^2 = 22.364, $p \leq 0.001$). Response rate across the follow-ups was: 73.2%, 57.5%, 53%, 50.8%, 43.9% and 34.3%.

TABLE 1 | Description of sociodemographic variables for the total sample and as a function of sex.

Age	Total sample	Women (n = 782)	Men (n = 458)
Mean	19.14 ± 1.7	19.06 ± 1.68	19.29 ± 1.74
18–19	73.4	76.1	68.8
20–21	15	13.1	18.3
22–23	7.8	6.9	9.4
24–25	3.8	3.9	3.5
Province of origin			
Córdoba	71.9	72.9	70.1
Other	28.1	27.1	29.9
Career			
Economy	20.8	21.2	20.1
Psychology	13.9	18.3	6.3
Engineering	16.9	8.3	31.7
Medical sciences	11	13.5	6.8
Philosophy	9.5	9.6	9.9
Odontology	8.8	10.1	6.6
Other	19.1	19.1	18.6

For categorical variables, values correspond to the percentage of participants placed in each category. For continuous variables, data are presented as means ± standard deviation.

Procedure

Thirteen departments of the National University of Cordoba (UNC, Argentina) received a brief invitation containing a description of the study and its aims. The National University of Cordoba (UNC, Argentina) began its activity in 1613 and is the oldest and one of the largest (approximately, 110,000 undergraduate students) universities of Argentina. Due to the central location of Cordoba City in Argentina, UNC attracts high-school graduates from different states of the country. The majority of the students were, however, from the Cordoba state and, particularly, from the city of Cordoba. These individuals belong to middle- and upper-middle-class families of large and medium-sized production farmers, professionals, and local merchants.

Ten departments accepted and authorized the research team to collect data in the classrooms. The researchers, in a face-to-face event, invited the students to participate in the study and explained its general aim, i.e., to better understand substance use behaviors during the college years, *via* a longitudinal study. Researchers also explained that participation involved completing a paper and pencil survey and several on-line follow-up surveys (six follow-ups during 3 years), which required providing contact information (e.g., email address). Students were informed about voluntary participation and anonymity. All procedures were approved by the university's internal review board, and the protocol was reviewed by the National Agency for Promotion of Science and Technology.

Measures

Alcohol Use

Following previous work (Pilatti et al., 2014), we used an *ad hoc* designed questionnaire to assess alcohol use in all data collections. We defined alcohol use as the consumption of at least one glass (i.e., 250 ml) of any alcoholic beverage. Participants indicated: (1) the two most consumed alcoholic beverages (e.g., beer, wine, vermouth, and so on); (2) usual frequency of alcohol use (i.e., from less than once per month to ≥4 times per week); and (3) number of glasses (a standard size of glass was provided) consumed per drinking occasion (i.e., from 1 glass to ≥14 glasses). Those who have never drunk alcohol or who abstained from drinking within the previous year had an option to indicate so. Based on known alcoholic contents in each alcoholic beverage, answers to questions 1 and 3 were used to calculate the grams of alcohol consumed per drinking occasion. To estimate the total volume of alcohol consumed within a month, we calculated the product of usual frequency (i.e., the number of drinking days within a month) by the grams of alcohol consumed per drinking occasion. Both grams per drinking occasion and total volume were considered continuous variables. Participants also indicated usual frequency (i.e., from less than once per month to ≥4 times per week) of HED (i.e., ≥4/5 standard drinks (women/men) in a single drinking occasion (as defined by the Ministerio de Salud Argentino, 2012). The questionnaire provided, to

facilitate the identification of HED, examples of the quantity of alcoholic beverages that corresponds to a heavy drinking episode (e.g., 6/7 glasses of beer or 4/5 glasses of wine for women and men respectively). Previous work examined the correspondence between estimates of alcohol consumption obtained with this *ad hoc* retrospective questionnaire with those obtained through an alcohol consumption diary (Pilatti et al., submitted). Results showed a large correspondence between those estimates, providing evidence on the validity of these retrospective estimates of alcohol consumption. Finally, students were asked to report: (i) the age they first drank one standard drink or more of any alcoholic beverage; and (ii) the occurrence of drunkenness episodes in their lifetime.

Alcohol-Related Negative Consequences

We used the Spanish version (S-YAACQ; Pilatti et al., 2016) of the Young Adult Alcohol Consequences Questionnaire (YAACQ; Read et al., 2006) to measure 48 alcohol-related negative consequences. Participants indicated whether or not they had experienced each consequence within each period. In the first data collection the timeframe was the previous year, and in the remaining surveys was the previous 3 months. The total score reflects the total number of consequences that the individual has experienced and, thus, was considered a continuous variable. The internal consistency, estimated with the tetrachoric correlation coefficient (Ledesma et al., 2011), was 0.90. Although this questionnaire was included in the first, third, fourth, sixth, and seventh data collection; the results described in the present study correspond to the first and last data collection.

Impulsivity

We used the Spanish version (Verdejo-García et al., 2010) of the UPPS-P, which features 59 items to measure five dimensions of impulsivity: Positive Urgency ($\alpha = 0.90$), Negative urgency ($\alpha = 0.84$), Lack of Premeditation ($\alpha = 0.83$), Lack of Perseverance ($\alpha = 0.78$) and Sensation Seeking ($\alpha = 0.84$). The items are scored on a 4-point scale, ranging from 1 (strongly agree) to 4 (strongly disagree). Items of each sub-scale are summed up and higher scores indicate higher impulsivity levels. Scores in each sub-scale are treated as continuous variables. Impulsivity was measured during the second data collection.

Stressful Life Events

The Inventory of Stressful Vital Events (Inventario de Acontecimientos Vitales Estresantes, AVE, Oliva et al., 2008) was used. The inventory consists of 29 items that evaluate stressful life events experienced during the last year, in different contexts (e.g., family, academic or work environment, peer group, etc.). Affirmative responses are summed; thus, the total score reflects the total number of stressful events that the individual has experienced. The total score is treated as a continuous variable. The internal consistency, estimated with the tetrachoric correlation coefficient (Ledesma et al., 2011), was 0.78. This questionnaire was included during the second data collection.

Descriptive Social Norms for Alcohol Use

Based on Pilatti et al. (2013), we asked the perceived frequency of alcohol use for the closest female and male friend (i.e., from less than once per month to $\geq$4 times per week). Those whose friends did not drink alcohol had an option to indicate so. This question allowed calculating the friend's number of days with alcohol consumption per month (continuous variable). Descriptive social norms for alcohol use were measured during the first data collection.

Family History of Alcohol Use Disorders

Following LaBrie et al. (2010), participants reported if a first (mother or father), second (siblings or grandparents) or third-degree relative (uncles) has or has had a history of alcohol abuse that derived, or could have derived, in treatment. Those reporting a family member with those characteristics were considered FH+ and those who did not were considered FH−. Family history of alcohol use disorders was measured during the first data collection.

Analysis Strategy

Latent Class Growth Analysis (LCGA) was performed to identify the HED trajectories that best fit the data. LCGA allows identifying qualitatively different groups or classes (Muthén and Muthén, 2000) that exhibit prototypical patterns of change over time, for an outcome of interest. Thus, individuals within each class differ in the level of the variable of interest (e.g., frequency of HED) at the beginning of the time window examined (intercept) and at the pattern of change (slope) over time (McArdle and Nesselroade, 2003). LCGA allows individuals with missing data to still contribute to the estimation of model parameters. Trajectories were estimated using seven data points per individual representing the usual frequency of HED (0 = no HED, 1 = less than monthly, 2 = monthly, 3 = two to three times per month, 4 = weekly, 5 = two times per week and 6 = three or more times per week).

LCGA models with two to seven latent classes were sequentially tested to determine the best fitting and most parsimonious model. The best-fitting model was selected based on interpretability and theoretical relevance of the classes, and on several statistical criteria: Akaike information criterion (AIC; Akaike, 1987), Bayesian information criterion (BIC; Sclove, 1987), entropy values and Lo-Mendell-Rubin adjusted likelihood ratio tests (LMR-LRT, Lo et al., 2001). To ensure that the latent classes capture a meaningful portion of the sample, each class had to include at least 5% of the sample (Nagin and Tremblay, 2005). We then applied one-way ANOVAs to several alcohol use indicators (grams consumed per drinking occasion, the volume of alcohol consumed within a month and negative consequences experienced—calculated only among last-year drinkers), with the identified trajectories as between factor, at the beginning (Time 1) and end (Time 7) of the examined period.

Once participants had been assigned to a trajectory group, multinomial logistic regression (MLR) was used to identify which variables predicted group membership. MLR explores how a set of variables distinguishes between different categories of a categorical dependent measure (i.e., the HED trajectories). The independent variables, all measured during the first or second data collection, were the age of drinking onset, alcohol intoxication (yes/no), the five dimensions of trait impulsivity

(according to UPPS-P), best female and male friend perceived drinking frequency, stressful life events experienced and family history of alcohol use disorders (yes/no). The odds ratios (ORs) and 95% confidence intervals (CI) were estimated. The ANOVAs and MLR were performed using SPSS 20.0, whereas LCGA was run in Mplus version 6.12. Alpha value was set at 0.05. The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

Identification and Description of HED Trajectories

The six-class model was selected. The BIC, AIC, LMR-LRT *p*-value, and entropy values for the different models are shown in **Table 2**. The BIC and AIC values continued to decrease with an increasing number of classes. However, the entropy value, the posterior probabilities, the LMR-LRT adjustment index, and the theoretical relevance of the classes favored the six-class model. **Figure 1** shows the adjusted growth curves for the six HED trajectories identified and **Table 3** shows the precision of latent class assignment, the estimated class proportions, and the Intercept and Slope values for each class.

Based on the shape of the growth curves, the classes were labeled: (1) *Heavy Stable Frequency* -HSF- (n = 116, 9.4%) included those who drank the heaviest (i.e., reported HED almost twice a week) consistently over the 3 years; (2) *Moderate Stable Frequency* -MSF- (n = 373, 30.1%) included those who had a moderate HED (i.e., 2–4 per month) across the 3 years; (3) *Moderate Decreasing Frequency* -MDF- (n = 88, 7.1%) included those who began the study with moderate levels of drinking (i.e., HED once per week), but declined as the study progressed; (4) *Stable Infrequent* -SI- (n = 274, 22.1%) included those who had HED less than monthly consistently over the 3 years; (5) *Decreasing Infrequent* -DI- (n = 156, 12.6) included those who began the study having heavy drinking episodes less than monthly, but declined as the study progressed; and (6) *No-HED* (n = 233, 18.8%) included those that did no exhibit HED over the timeframe examined.

Differences in Alcohol Use Between the HED Trajectories

At Time 1, the HED trajectories significantly differed in the grams of alcohol consumed per drinking occasion ($F_{(1236)}$ = 179.42, $p < 0.001$), the monthly volume of alcohol consumed ($F_{(1239)}$ = 190.64, $p < 0.001$) and the number of negative consequences reported ($F_{(1147)}$ = 102.44, $p < 0.001$). Mean values for each class are presented in **Table 4**. The *post hoc* analysis revealed, for both grams per drinking occasion and alcohol-related negative consequences, significantly greater mean values in the *Heavy Stable Frequency* class when compared to the *Moderate Stable Frequency* or the *Moderate Decreasing Frequency* class. The latter two classes scored significantly higher than the *Stable Infrequent/Decreasing Infrequent* classes, which in turn scored significantly higher than the *No-HED* class. For the volume of alcohol consumed within a month, the *post hoc* analyses revealed greater mean values in the *Heavy Stable Frequency* than in the *Moderate Decreasing Frequency* class. The latter class scored significantly higher than the *Moderate Stable Frequency* class, which in turn scored significantly higher than the *Stable Infrequent* and *Decreasing Infrequent* classes. The *No-HED* scored significantly lower than the rest of the classes.

At Time 7, the grams of alcohol consumed per drinking occasion ($F_{(633)}$ = 89.57, $p < 0.001$), the monthly volume of alcohol consumed ($F_{(633)}$ = 113.69, $p < 0.001$) and the negative consequences ($F_{(581)}$ = 28.68, $p < 0.001$) significantly

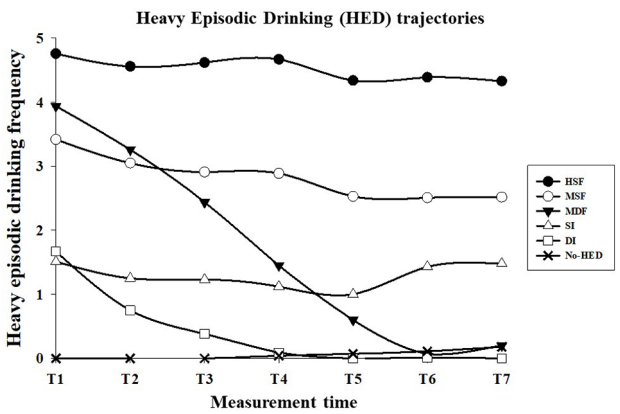

FIGURE 1 | Trajectories of heavy episodic drinking (HED) frequency since college entry and during the first 3 years of college. Frequency of HED: 0 = no HED, 1 = less than monthly, 2 = monthly, 3 = 2–3 times per month, 4 = weekly, 5 = 2 times per week, and 6 = 3 or more times per week. HSF, Heavy Stable Frequency, 9%; MSF, Moderate Stable Frequency, 30%; MDF, Moderate Decreasing Frequency, 7%; SI, Stable Infrequent, 22%; DI, Decreasing Infrequent, 13%; No-HED, without HED, 19%.

TABLE 2 | Fit statistics for the different latent growth models.

Number of classes	AIC	BIC	aBIC	Entrophy	LMR-LRT *p*-value
2	19,387.373	19,438.602	19,406.837	0.835	0.000
3	18,724.566	18,791.163	18,749.869	0.826	0.001
4	18,505.477	18,587.443	18,536.620	0.767	0.000
5	18,394.378	18,491.713	18,431.360	0.758	0.004
6	**18,319.658**	**18,432.361**	**18,362.480**	**0.740**	**0.000**
7	18,290.236	18,418.308	18,338.897	0.700	0.133

AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion; aBIC, sample-size adjusted Bayesian Information Criterion; LMR-LRT, Lo-Mendell-Rubin adjusted LRT test. The selected model is indicated in bold font.

TABLE 3 | Growth factor parameter estimates and posterior probabilities for the 6-Class model.

Classes	Class proportion	Classification accuracy	Intercept	Slope
Class 1	0.13	0.80	−3.489	−1.003***
Class 2	0.09	0.89	1.599	−0.138*
Class 3	0.30	0.83	−0.901	−0.201***
Class 4	0.19	0.86	−9.625	0.580**
Class 5	0.22	0.79	−3.463	−0.055
Class 6	0.07	0.70	0.000	−1.130***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

differed as a function of the class. For grams of alcohol consumed per drinking occasion, the pattern of observed differences were as follows: *Heavy Stable Frequency* > *Moderate Stable Frequency* > *Stable Infrequent* > *Moderate Decreasing Frequency/Decreasing Infrequent/No-HED*. For the volume of alcohol consumed within a month, the *post hoc* analysis revealed that *Heavy Stable Frequency* > *Moderate Stable Frequency* > *Stable Infrequent* > *Decreasing Infrequent/No-HED*. The *Moderate Decreasing Frequency* trajectory was significantly different from all the classes, except for the *Stable Infrequent* and *Decreasing Infrequent/No-HED* trajectories. The *post hoc* tests conducted for the number of alcohol-related negative consequences showed greater mean values in the *Heavy Stable Frequency* class when compared to the *Moderate Stable Frequency* or the *Stable Infrequent* classes. The latter two classes scored significantly higher than the *Decreasing Infrequent* and *No-HED* classes. *Moderate Decreasing Frequency* trajectory located in an intermediate position between the *Stable Infrequent* and *Decreasing Infrequent* trajectories, without showing significant differences from these trajectories but significantly differing from the others (mean values presented at **Table 4**).

Predictors of HED Trajectories

The Deviance criterion ($\chi^2_{(4,952)}$ = 2809.73, p = 1.00) indicated a good model fit (discrimination among groups) based on 11 variables. The model explained 43% of the variance (Nagelkerke's Pseudo R^2 = 0.435). Likelihood ratio tests showed six of the variables were significant predictors of class membership (age of drinking onset, drinking intoxication, lack of premeditation -PREM-, positive urgency -PU-, sensation-seeking -SS- and perceived drinking frequency of the best same-sex friend). Correct classification rates using these variables were 76.8% for *Moderate Stable Frequency*, 68.2% for *No-HED*, 27.3% for *Stable Infrequent*, 4.5%, and 21.7% for *Decreasing Infrequent* and *Heavy Stable Frequency*, respectively. There was no correct classification for the *Moderate Decreasing Frequency* group. The overall correct classification rate was 44.3%.

Table 5 reports ORs and 95% CIs for the variables included in the model, comparing the *No-HED* trajectory vs. the others. Younger age of drinking onset (OR = 0.59 and OR = 0.67 for *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories, respectively), alcohol intoxication (OR = 156.55 and OR = 43.43 for *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories, respectively), greater sensation seeking (OR = 1.06 and OR = 1.05 for *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories, respectively), greater positive urgency (OR = 1.09 and OR = 1.07 for *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories, respectively) and a perception of a greater frequency of alcohol use among same-sex peers (OR = 1.33 and OR = 1.14 for *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories, respectively) predicted membership to the trajectories with greater HED, compared to the *No-HED* trajectory. Considering the *Moderate Decreasing Frequency* trajectory as the comparison group, alcohol intoxication was significantly associated to the *Heavy Stable Frequency* (OR = 18.29) and *Moderate Stable Frequency* (OR = 5.07) trajectories, and lower levels of lack of premeditation were associated to the *Decreasing Infrequent* (OR = 0.91), *Stable Infrequent* (OR = 0.92) and *No-HED* (OR = 0.92) trajectories (results in **Table 6**).

DISCUSSION

This study identified, in the understudied population of Argentinean college students, six HED trajectories during the first 3 years of college: a trajectory with frequent and stable HED (*Heavy Stable Frequency*); two trajectories with moderate HED at the beginning of the examined period, one that remained stable over time (*Moderate Stable Frequency*) and one with a marked decrease in HED frequency over time *(Moderate Decreasing Frequency*); two trajectories with infrequent HED, one that remained stable over time (*Stable Infrequent*) and one with a marked decrease in HED frequency over time (*Decreasing Infrequent*); and, finally, a trajectory with no HED or very low frequency (*No-HED*). These trajectories showed differences in alcohol use. Notably, the *Heavy Stable Frequency* and *No-HED* trajectories seemed to present the greatest and lowest risk, since their members exhibited the greatest and lowest levels of alcohol consumption and alcohol-related negative consequences, respectively.

The identified trajectories partially coincide with those found in studies from other cultures. Similar to other studies (Tucker et al., 2003; Goudriaan et al., 2007; Jackson et al., 2008; Sher et al., 2011; Ashenhurst et al., 2015), we identified the high and stable frequency trajectory, the low and stable frequency trajectory, a near-zero frequency trajectory, a moderate and stable frequency trajectory and two decreasing frequency trajectories that had different levels of alcohol consumption at the beginning of the study.

Unlike previous studies (Chassin et al., 2002; Jackson et al., 2008; Sher et al., 2011; Ashenhurst et al., 2015), we did not

TABLE 4 | Alcohol use indicators as a function of class membership during Time 1 and Time 7.

	HSF		MSF		MDF		SI		DI		No-HED	
	Time 1	Time 7	Time 1	Time 7	Time 1	Time 7	Time 1	Time 7	Time 1	Time 7	Time 1	Time 7
Grams per drinking occasion	219.96 ± 10.02	146.26 ± 10.01	156.38 ± 4.47	102.4 ± 4.25	159.82 ± 9.18	44.77 ± 5.78	88.32 ± 3.67	68.78 ± 3.12	77.88 ± 3.91	28.15 ± 2.55	21.24 ± 1.37	22.11 ± 2.27
Usual Frequency	6.92 ± 0.3	7.07 ± 0.54	3.65 ± 0.12	3.32 ± 0.19	4.22 ± 0.29	2.27 ± 0.45	1.92 ± 0.08	2.61 ± 0.17	2.01 ± 0.14	1.52 ± 0.24	0.81 ± 0.05	1.01 ± 0.12
Volume of alcohol	17, 51.87 ± 122.83	968.49 ± 81.92	620.21 ± 27.11	349.47 ± 22.06	809.89 ± 80.57	115.49 ± 18.72	194.08 ± 10.37	186.18 ± 13.46	195.94 ± 18.82	70.53 ± 47.56	33.92 ± 3.18	44.89 ± 6.59
Drunkenness episodes	0.53 ± 0.07	1.23 ± 0.31	0.26 ± 0.02	0.58 ± 0.08	0.25 ± 0.05	0.21 ± 0.09	139 ± 0.02	0.4 ± 0.08	0.06 ± 0.02	0.05 ± 0.03	–	0.13 ± 0.04
Negative consequences	14.51 ± 0.74	12.43 ± 1.31	11.22 ± 0.33	7.06 ± 0.44	10.1 ± 0.66	4.93 ± 1.46	6.75 ± 0.30	5.38 ± 0.47	5.89 ± 0.4	1.64 ± 0.29	1.78 ± 0.21	1.47 ± 0.38

Data are presented as means ± standard errors of the mean. HSF, Heavy Stable Frequency; MSF, Moderate Stable Frequency; MDF, Moderate Decreasing Frequency; SI, Stable Infrequent; DI, Decreasing Infrequent; No-HED, without Heavy Episodic Drinking.

find a trajectory with increasing HED frequency. This could relate to the fact that alcohol consumption was measured at a time when all students had already reached their peak in alcohol use. The average age of the students at the beginning of this study was 19.14 ± 1.7 years old, roughly one more year than those college students in the studies that identified an increasing alcohol use trajectory (Greenbaum et al., 2005; Sher et al., 2011; Ashenhurst et al., 2015). Also, in Argentina, the minimum legal age to buy alcohol is 18 years old, which means that the participants of our study had already legal access to buy alcohol at the beginning of the measurement. This, in turn, may affect the age when the peak in alcohol consumption is achieved. Supporting this idea, it has been found that, in the United Kingdom, where the legal age to buy alcohol is 18 years old, the peak in alcohol consumption occurs between 18–19 years old (Bewick et al., 2008), while in the U.S., where the legal age to buy alcohol is 21 years old, it has been usually registered between 21–22 years of age (Chen and Jacobson, 2012; Jager et al., 2015; Patrick et al., 2016). Finally, we need to remember that Argentinean students cannot affiliate to fraternities or sororities nor live on campus, all of which has been associated with heavier alcohol use (Barry, 2007; Maggs et al., 2011; Lorant et al., 2013; White and Hingson, 2013). Instead, during their college years, most Argentinean college students continue living with their families under parental supervision, which could discourage heavy alcohol use (Evans-Polce et al., 2017; Patrick and Terry-McElrath, 2017).

Regarding risk factors that discriminated between the identified trajectories, the results showed that those with a younger age of drinking onset and whoever felt intoxicated had a markedly higher probability of belonging to the *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories than to the *No-HED* trajectory. These results coincide with those found in cross-sectional and prospective studies (Kuntsche et al., 2013; Moss et al., 2014; Jackson et al., 2015; Asbridge et al., 2016), where a lower age of drinking onset and alcohol intoxication has been associated with increased alcohol consumption, and also with trajectory studies in which early drinking onset increases the probability of belonging to a stable frequent drinking trajectory (Casswell et al., 2002). It should be noted that the effect of alcohol intoxication was greater than the effect of drinking onset. The present findings extend the conclusions of our previous analysis of the ELSA 2014 study (using data from the first-wave, Vera et al., 2020) where we examined the interactive effects of age of drinking onset and progression to drunkenness (i.e., time—in years—elapsed between the first contact with alcohol and the first episode of alcohol intoxication) on alcohol outcomes. In that study, we found that alcohol intoxication seems to kindle the expression of the vulnerability associated with early drinking onset, so that the early drinking onset promote the greater occurrence of alcohol-related negative consequences only among those who ever got drunk, while drunkenness naïve participants seem to be insensitive to the promoting effect of early-onset on alcohol consequences. That is, the age of drinking onset seems to be no longer relevant when there is no alcohol intoxication. Although more studies are needed, these results imply that reducing or impeding drunkenness is a valuable

TABLE 5 | Multinomial logistic regressions comparing No-HED trajectory vs. the others: odds ratio estimates.

	Heavy Stable Frequency vs. No-HED			Decreasing Infrequent vs. No-HED			Stable Infrequent vs. No-HED			Moderate Decreasing Frequency vs. No-HED			Moderate Stable Frequency vs. No-HED		
	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
		LB	UB		LB	UB		LB	UB		LB	UB		LB	UB
Age Onset	**0.59**	0.47	0.73	0.90	0.77	1.06	**0.78**	0.67	0.91	**0.67**	0.54	0.84	**0.67**	0.57	0.74
Intoxication	**156.55**	19.24	1,273.90	**5.75**	3.45	9.59	**12.36**	7.39	20.69	**8.56**	3.95	18.54	**43.43**	21.44	87.98
UN	1.02	0.96	1.08	0.96	0.92	1.01	1.01	0.96	1.06	1.00	0.94	1.06	0.99	0.95	1.04
PREM	1.06	0.99	1.14	0.98	0.93	1.04	1.00	0.94	1.05	**1.08**	1.01	1.16	1.04	0.98	1.10
PERSEV	0.99	0.92	1.06	1.03	0.97	1.09	1.00	0.95	1.06	1.01	0.94	1.09	0.98	0.93	1.04
SS	**1.06**	1.02	1.11	1.00	0.96	1.03	1.02	0.99	1.06	1.03	0.98	1.07	**1.05**	1.01	1.08
PU	**1.09**	1.03	1.15	**1.06**	1.00	1.11	1.02	0.97	1.07	1.05	0.99	1.11	**1.07**	1.02	1.12
Same-sex drinking	**1.33**	1.18	1.47	1.05	0.94	1.17	1.03	0.93	1.14	1.12	0.99	1.27	**1.14**	1.03	1.26
Opposite-sex drinking	1.02	0.92	1.11	0.97	0.89	1.06	0.99	0.91	1.07	1.00	0.90	1.11	0.95	0.88	1.04
FH+	0.78	0.40	1.52	0.73	0.43	1.26	0.60	0.36	0.99	0.86	0.45	1.68	0.50	0.30	0.84
Stressful events	0.97	0.89	1.05	0.97	0.91	1.04	0.99	0.94	1.05	1.00	0.92	1.08	0.99	0.93	1.05

OR, odds ratios; CI, confidence intervals; LB, lower bound; UB, upper bound; UN, negative urgency; PREM, lack of premeditation; PERS, lack of perseverance; SS, sensation seeking; PU, positive urgency; FH+, family history of alcohol abuse. Significant odd ratios are indicated in bold font.

TABLE 6 | Multinomial logistic regressions comparing the Moderate Decreasing Frequency trajectory vs. the others: odds ratio estimates.

	Heavy Stable Frequency vs. MDF			Decreasing Infrequent vs. MDF			Stable Infrequent vs. MDF			Moderate Stable Frequency vs. MDF		
	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
		LB	UB		LB	UB		LB	UB		LB	UB
Age Onset	0.87	0.68	1.11	**1.34**	1.08	1.67	1.16	0.95	1.42	0.99	0.81	1.21
Intoxication	**18.29**	2.09	159.67	0.67	0.30	1.51	1.44	0.65	3.22	**5.07**	2.02	12.75
UN	1.02	0.96	1.08	0.96	0.91	1.02	1.01	0.96	1.07	0.99	0.94	1.04
PREM	0.98	0.91	1.05	**0.91**	0.85	0.98	**0.92**	0.87	0.98	0.96	0.91	1.02
PERSEV	0.98	0.91	1.06	1.02	0.95	1.09	0.99	0.93	1.06	0.98	0.92	1.04
SS	1.04	0.99	1.09	0.97	0.93	1.01	1.00	0.96	1.04	1.02	0.98	1.06
PU	1.04	0.99	1.10	1.01	0.96	1.07	0.97	0.93	1.02	1.02	0.97	1.07
Same-sex drinking	**1.18**	1.05	1.33	0.93	0.82	1.06	0.91	0.81	1.02	1.01	0.91	1.13
Opposite-sex drinking	1.01	0.91	1.13	0.97	0.87	1.08	0.99	0.90	1.09	0.95	0.87	1.04
FH+	0.90	0.44	1.82	0.85	0.44	1.64	0.69	0.38	1.26	0.58	0.32	1.04
Stressful events	0.97	0.89	1.06	0.98	0.90	1.06	1.00	0.93	1.07	0.99	0.93	1.06

OR, odds ratios; CI, confidence intervals; LB, lower bound; UB, upper bound; UN, negative urgency; PREM, lack of premeditation; PERS, lack of perseverance; SS, sensation seeking; PU, positive urgency; FH+, family history of alcohol abuse. Significant odd ratios are indicated in bold font.

intervention milestone for those that have already begun to use alcohol.

Another relevant factor was the perceived frequency of alcohol use among same-sex peers. In general, a greater perception of peer drinking frequency increased the probability of belonging to the trajectories with more frequent HED. These results coincide with those found in adolescents (Chassin et al., 2002; van der Vorst et al., 2009; Danielsson et al., 2010) and emerging adults (Lee et al., 2012). It should be noted that the results suggest that the effect is specific for the drinking perception of same-sex peers. Previous studies have indicated that the strength of the association between perceived social norms and alcohol use depends on the specification and closeness of the reference group (Neighbors et al., 2010; Collins and Spelman, 2013). Usually, this association is stronger when the social norms derive from those close to the drinker (e.g., stronger with a best friend instead of the typical college student), compared to when the social norms derive from more distal social referents (Lewis and Neighbors, 2004; Collins and Spelman, 2013). Here, we found significant relations only when including perceived drinking norms of same sex-peers.

As in previous studies (Del Boca et al., 2004; Jackson et al., 2008; Ashenhurst et al., 2015), higher levels of sensation-seeking and positive urgency increased the probability of belonging to the trajectories with more frequent HED. These effects were small, which was not unexpected, given that distal variables tend to have smaller effects than proximal variables.

The *Moderate Decreasing Frequency* trajectory exhibited an intriguing pattern. The members of this trajectory drank, at the beginning of the study, similarly to those of the *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories. However, by the end of the study, they reduced their consumption to that exhibited by the *Stable Infrequent*, *Decreasing Infrequent*, and *No-HED* trajectories members. Interestingly, the only risk factor that allowed distinguishing between this trajectory and the *Heavy Stable Frequency* and *Moderate Stable Frequency* trajectories was alcohol intoxication. Thus, yet again lifetime

drunkenness explains why, over time and despite exhibiting similar levels of alcohol use at the beginning, some participants kept stable levels of HED whereas others decreased this pattern of alcohol consumption.

A limitation of the study was the relatively high level of data attrition. Moreover, the drinking patterns of those who dropped out of the study were significantly different from those who kept participating. We can only speculate, yet it is possible that those who left the study may have endorsed trajectories with high HED frequency, had they continued participating. However, it is unlikely that this would have changed the quantity or the shape of the identified trajectories. Another limitation is the reliance on self-reported data, which might be affected by the participants' ability to recall, which is sensible to telescoping/extrapolating biases. A non-probabilistic sampling was also used, thus limiting the possibility of generalizing the results to all Argentinean college students. The study also lacks the measurement of neurobiological characteristics. Recently, a preclinical study (Siciliano et al., 2019) showed that the activation of neurons projecting from the medial prefrontal cortex to the periaqueductal gray area predicted the emergence of risky alcohol drinking, after a phase of binge alcohol exposure. It has been also shown that some individuals are particularly sensitive to reward-associated cues, including those associated with alcohol (Versaggi et al., 2016). Hence, these individuals may be particularly prone to the effects of these stimuli on craving or relapse to drug-seeking behavior. Future extensions of the present study should consider adding these neurobiological measures, or appropriate proxies, to enhance the predictive ability of the model.

Despite these limitations, the study contributes towards identifying drinking trajectories among college students of an understudied sociocultural context. This work represents a step forward in the identification of risk factors differentiating between the HED trajectories. Although more research is still needed, the results found in this study suggest that alcohol intoxication is an important explanatory factor of alcohol consumption among Argentinean college students and a likely intervention target. Thus, the study has implications for the design of interventions aimed at detecting students at risk for engaging in problematic alcohol drinking. Such early detection at the individual level, which could also look for students featuring high levels of impulsivity and early age of first alcohol use, should be undertaken early in the student's academic trajectory and complemented with campus-wide preventive actions, likely focusing in reducing normative perceptions on alcohol use.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National University of Córdoba (Argentina) internal review board, and the protocol was reviewed by the National Agency for Promotion of Science and Technology of Argentina. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The results presented are part of BV's Ph.D. work. AP and RP designed the study and helped with data collection and supervised data analysis. BV organized the database, performed the statistical analysis, and wrote the first draft of the manuscript and subsequent versions. AP and RP edited the first draft of the manuscript and subsequent versions of it. All authors read, corrected, and approved the final submitted version.

FUNDING

This work was supported by grants from the National Secretary of Science and Technology (FONCYT 2015-849) and the Secretary of Science and Technology-National University of Córdoba (SECyT-UNC) to AP. FONCyT and SECyT-UNC had no role in the study design, collection, analysis, and interpretation of the data, writing the manuscript, or the decision to submit the article for publication.

ACKNOWLEDGMENTS

The authors are grateful to all the participants who participated in the study. We would also like to show our gratitude to Florencia Caneto, Francisco Tuzinkievich, Oscar Lagoria, Virginia Moroni and Denise Roqué for their assistance during data collection and Oscar Lagoria, Virginia Moroni, Denise Roqué and María Micaela Marín for their assistance during data entry.

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 19 June 2020
doi: 10.3389/fnbeh.2020.00092

Anxious Behavior of Adult CD1 Mice Perinatally Exposed to Low Concentrations of Ethanol Correlates With Morphological Changes in Cingulate Cortex and Amygdala

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OPEN ACCESS

Edited by:
Ricardo Marcos Pautassi, Universidad Nacional de Córdoba, Argentina

Reviewed by:
Waldo Cerpa, Pontificia Universidad Católica de Chile, Chile
María Pascual, University of Valencia, Spain

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Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *15 December 2019*
Accepted: *14 May 2020*
Published: *19 June 2020*

Citation:
Madarnas C, Villalba NM, Soriano D and Brusco A (2020) Anxious Behavior of Adult CD1 Mice Perinatally Exposed to Low Concentrations of Ethanol Correlates With Morphological Changes in Cingulate Cortex and Amygdala. Front. Behav. Neurosci. 14:92. doi: 10.3389/fnbeh.2020.00092

Perinatal ethanol (EtOH) exposure is associated with high incidence of behavioral disorders such as depression and anxiety. The cerebral areas related with these consequences involve the corticolimbic system, in particular the prefrontal cortex, hippocampus, amygdala, and cingulate cortex, although the latter has not been thoroughly studied yet. Different animal models of prenatal or perinatal EtOH exposure have reported morphofunctional alterations in the central nervous system, which could explain behavioral disorders along life; these results focus on youth and adolescents and are still controversial. In the light of these inconclusive results, the aim of this work was to analyze adult behavior in CD1 mice perinatally exposed to low concentrations of EtOH (PEE) during gestation and lactation, and describe the morphology of the cingulate cortex and amygdala with a view to establishing structure/function/behavior correlations. Primiparous CD1 female mice were exposed to EtOH 6% v/v for 20 days prior to mating and continued drinking EtOH 6% v/v during pregnancy and lactation. After weaning, male pups were fed food and water *ad libitum* until 77 days of age, when behavioral and morphological studies were performed. Mouse behavior was analyzed through light–dark box and open field tests. Parameters related to anxious behavior and locomotor activity revealed anxiogenic behavior in PEE mice. After behavioral studies, mice were perfused and neurons, axons, serotonin transporter, 5HT, CB1 receptor (CB1R) and 5HT1A receptor (5HT1AR) were studied by immunofluorescence and immunohistochemistry in brain sections containing cingulate cortex and amygdala. Cingulate cortex and amygdala cytoarchitecture were preserved in adult PEE mice, although a smaller number of neurons was detected in the amygdala. Cingulate cortex axons demonstrated disorganized radial distribution and reduced area. Serotonergic and endocannabinoid systems, both involved in anxious behavior, showed differential expression. Serotonergic afferents were lower in both brain areas of PEE animals, while 5HT1AR expression was lower in the cingulate cortex and higher in the amygdala. The expression of CB1R was lower only in the amygdala. In

sum, EtOH exposure during early brain development induces morphological changes in structures of the limbic system and its neuromodulation, which persist into adulthood and may be responsible for anxious behavior.

Keywords: perinatal, ethanol, cingulate cortex, amygdala, behavior, adult, anxious behavior

INTRODUCTION

Maternal alcohol consumption produces a spectrum of deleterious effects on offspring whose incidence is around 10% in the general population (Popova et al., 2017). Fetal alcohol spectrum disorders (FASD) encompass a range of pathological conditions resulting from alcohol consumption of different magnitudes and during different stages of pregnancy (Riley and McGee, 2005; Hoyme et al., 2016), which include cognitive, behavioral, and adaptive functional deficits (Mattson et al., 2019). Attention has focused on FASD as a serious public health issue and has encouraged research into the basic mechanisms of prenatal alcohol exposure and its long-term consequences (Koren and Navioz, 2003).

As part of FASD, the fetal alcohol syndrome (FAS) represents one of the most severe conditions. Several studies have examined the extent to which the frequency and severity of FAS are related to the amount of alcohol consumed and the temporal pattern of consumption (Sayal et al., 2009; May et al., 2011, 2013). Ethanol (EtOH) exposure during pregnancy has been then found to cause serious morphological, behavioral, and cognitive alterations in developing children, which may also persist into adulthood (Mattson et al., 2001; Lebel et al., 2011; Donald et al., 2016; Hoyme et al., 2016). Neurobehavioral impairment has been documented not only in FAS children severely exposed to EtOH but also in children prenatally exposed to moderate EtOH doses (O'Connor and Paley, 2006; Murray et al., 2016). Moreover, alcohol abuse prior to pregnancy may have persistent adverse effects that are not obliterated by abstinence during pregnancy.

Studies in both humans and animals have extensively demonstrated the deleterious effects of maternal alcohol ingestion on the fetus (Aronne et al., 2008, 2011; Gil-Mohapel et al., 2010; Ornoy and Ergaz, 2010). In individuals affected by FASD, secondary alterations have been described, which mainly include mental health disorders (Barr et al., 2006; Weyrauch et al., 2017). Clinical studies suggest a correlation between prenatal EtOH exposure and the incidence of anxiety-related disorders during adolescence and adulthood (Barr et al., 2006). Additionally, it has also been reported that individuals with FASD present structural and functional alterations in different areas of the brain, among which structures belonging to the limbic system stand out (Mattson et al., 2001; Nardelli et al., 2011; Malisza et al., 2012; Roussotte et al., 2012; Yang et al., 2012; Wozniak et al., 2013; Donald et al., 2016). This system is involved in emotional processing and is formed by structures such as the amygdala, cingulate cortex, prefrontal cortex, and insula (Davidson et al., 2003). Alterations in these areas of the brain have been associated with numerous behavioral disorders, such as depression and anxiety (Aggleton and Brown, 1999; Dalgleish, 2004).

The effects of maternal alcoholism on offspring behavior have been widely studied in animal models, although the results reported are controversial and focus mainly on the periods of childhood and adolescence. Some authors have reported locomotor hyperactivity as one of the most characteristic effects of *in utero* EtOH intoxication, while others have found no hyperactivity (Abel and Berman, 1994; Tran et al., 2000; Downing et al., 2008; Brys et al., 2014).

It has also been observed that early exposure to alcohol—passive or in the context of operant learning schemes—alters consumption evaluated at later stages of development (Spear and Molina, 2005). Furthermore, a growing number of studies using rodents consistently demonstrate that prenatal EtOH exposure induces increased postnatal EtOH intake, as observed in studies in which EtOH was administered to the pregnant dam during most of gestation (Arias and Chotro, 2005; Youngentob et al., 2007; Aronne et al., 2013; Brancato et al., 2018).

The literature is also particularly controversial about anxiety-like behavior. While some authors have recorded an anxiogenic effect of *in utero* EtOH exposure (Hellemans et al., 2008; Cullen et al., 2013; Wille-Bille et al., 2018), others have reported a decrease in anxiety, even using similar animal treatments and models (Carneiro et al., 2005; Ohta et al., 2010; Diaz et al., 2016).

Indeed, a systematic review has recently shown the limited evidence available in the literature on the association between fetal alcohol exposure and offspring emotional problems in childhood or adolescence, in particular anxiety and depression (Easey et al., 2019). In the same way, animal model studies on adult offspring prenatally exposed to EtOH do not abound.

In this context, the aim of the present work was to determine the impact of alcohol exposure by analyzing animal behavior and the morphology of cingulate cortex and amygdala, two brain areas related to emotional behavior, in adult CD1 mice perinatally exposed to EtOH at low concentrations.

MATERIALS AND METHODS

Animals and Animal Care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of CICUAL, Facultad de Medicina, Universidad de Buenos Aires (Res. CD 2375/2017).

Twelve CD1 primiparous females (aged 45–50 days) and six adult CD1 males, all provided by the animal room

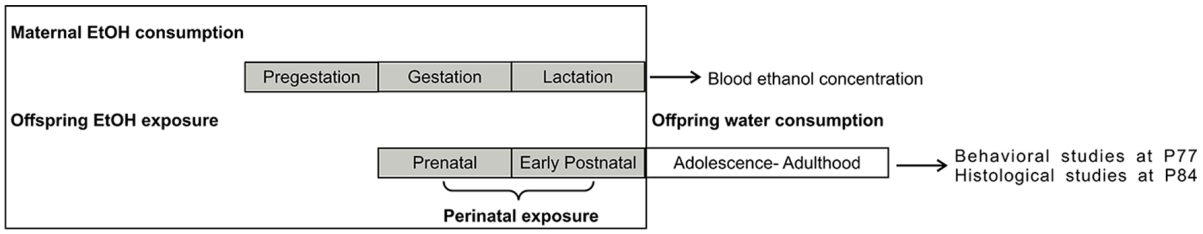

FIGURE 1 | Experimental procedure. E mothers consumed 6% v/v ethanol (EtOH) as the only beverage *ad libitum* during pregestational, gestational, and lactation periods. PEE pups were thus exposed to EtOH from conception to weaning, after which they drank water as the only beverage and had no further contact with EtOH. P, postnatal day.

at the Institute of Cell Biology and Neuroscience, were housed in cages (two females per cage and three males per cage) in a temperature (22–23°C) and photoperiod (12-h light/dark)-controlled room, with lights on between 08:00 and 20:00 h. Both the 12 females and the six males were randomly selected from different litters of the CD1 colony.

EtOH Exposure

Female mice were divided into two groups, a control group (C, six females) and an EtOH-exposed group (E, six females), and housed two in each cage. As from 20 days before mating, E female mice received a constant dilution of 6% v/v EtOH in water as the only beverage with standard food *ad libitum* until pup weaning. C female mice and all male mice received water and standard food *ad libitum*. One male mouse was put in each cage for mating, and pregnancy was determined by the detection of a vaginal plug (considered gestational day 0). Pregnant mice were separated, one per cage, for the rest of pregnancy and nursing. At postnatal day 1 (P1), all the litters were reduced to no more than 10 pups, preferentially male, to be used for the different studies. At P21, male offspring were separated from their mother and housed 3 to 6 per litter in each cage, with water and standard food *ad libitum* and no further contact with EtOH. Pups from E mothers were defined as the perinatally exposed to EtOH group (PEE) and the pups from C mothers the Control group. E mothers and PEE females were used for blood EtOH concentration (BEC) measurements at the end of lactation. C mothers and Control female pups were returned to complete their life as part of the colony, and male offspring of the two groups (Control and PEE) were submitted to behavioral studies at P77 and subsequently perfused for morphological measurements (**Figure 1**).

Dams' weight gain and beverage intake were controlled during pregestational, gestational, and lactation period. Once the litters were born, the number of offspring was counted and the pups' appearance was qualitatively evaluated. Maternal care behavior was qualitatively assessed three times a week during lactation period, which included observation of nest building, group care—not separating pups from the others—appropriated pups nursing, retrieving the pups to the nest when they were moved in cage changing, and being in contact with the pups. Also, male pups' body weight was registered at P21 and adulthood.

Blood EtOH Concentration

Post-weaning E dams and P21 PEE females were anesthetized to obtain blood samples from carotid arteries and later euthanized. The blood samples were collected in the light cycle, between 09:00 and 3:00 h, that is, 1–4 h after lights turn on according to authors who analyzed the peak of BEC in rodents (Simpson et al., 2005; Juarez et al., 2017). Both dam and P21 offspring BEC was determined in a spectrophotometer by means of an enzymatic method with a specific Quanti Chrom EtOH Assay Kit (Bioassay Systems).

Behavioral Studies of Adult CD1 Mice Perinatally Exposed to EtOH

Between P77 and P84, male PEE and Control pups corresponding to six different Control and PEE litters (see **Supplementary Table S1**) were tested for anxiety and locomotor activity in a behavioral test battery including the light–dark box test (LDB) followed by the open field test (OF). These two tests were conducted 1 week from one another and all animals performed the two test batteries in the specific order mentioned.

All tests were performed between 9:00 am and 2:00 pm, and animals were taken to the test room the day before at 5:00–6:00 pm. Once the three tests had finished, animals were returned to their housing room until morphological analyses.

LDB

The device used consists of two compartments (20 cm high, 20 cm wide, 15 cm deep) connected by a hole (4 cm wide and 5 cm high). Animals were placed in the light compartment, facing the hole. Animals were filmed for later video analysis and time spent in the light compartment and time spent in the dark compartment, and the number of transitions was registered during the next 5 min.

OF

The apparatus (50 cm wide, 50 cm long, 40 cm high) consists of an area with black plywood walls and wooden floor, divided into 16 squares by white lines (four central, 12 peripheral). The animals were put on the central area and were recorded with a video camera for 5 min for later analysis. Time spent in the central area, time in the peripheral area, latency, total

distance traveled, and number of rearings and thigmotaxis were measured.

Morphological Studies

After the behavioral test battery, 10 male mice per experimental group were randomly selected from all litters and deeply anesthetized with ketamine and xylazine in doses of 100 and 10 mg/kg, respectively. Animals were then perfused through the left ventricle, initially with physiological solution added to 50 IU heparin, and subsequently with a fixative solution containing 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed and postfixed in the same cold fixative solution for 4 h. Brains were then washed overnight in 5% (w/v) sucrose in PB at 4°C. Afterwards, brains were cryoprotected by immersion in a solution containing 30% (w/v) sucrose in PB and stored at −80°C until used. Coronal 50-μm-thick brain sections were cut using a cryostat (Leitz, Kryostat 1720 Digital), put in Eppendorf vials containing glycerol 50% in phosphate buffer saline (PBS), and stored at −20°C until used, or cut into 20-μm-thick brain sections and mounted directly on gelatin-coated slides. Brain sections corresponding to 1.10–0.02 mm Bregma level for anterior cingulate cortex (ACC) and −1 to −2.30 mm Bregma level for amygdala (Franklin and Paxinos, 2008) were processed for the corresponding histological studies.

Immunofluorescence

Five coronal 50-μm-thick brain sections containing ACC and amygdala were randomly selected from five mice per group, each one from different litters. Slices were washed three times in PBS and immersed in a solution of 3% (v/v) normal equine serum plus 0.5% (v/v) Triton X-100 in PBS for 3 h at 4°C under agitation to permeabilize and block unspecific sites. Sections were then incubated with the following primary antibodies diluted in a solution of 1% (v/v) normal equine serum and 0.3% (v/v) Triton X-100 in PBS: mouse anti-NeuN (mouse anti-neuronal nuclei, monoclonal antibody, 1:1,000, Millipore, Cat# MAB377, RRID:AB_2298772), rabbit anti-5HT1A receptor (5HT1AR; rabbit anti-serotonin receptor type 1A, polyclonal antibody, 1:1,000, Millipore, Cat# AB15350, RRID:AB_805421), rabbit anti-CB1 receptor (CB1R, rabbit anti-cannabinoid receptor type 1, 1:3,000, Cayman Chemicals, Cat# 10006590, RRID:AB_10098690), rabbit anti-5HT (rabbit anti serotonin, polyclonal antibody, 1:1,000, developed in our laboratory; Brusco et al., 1983), and mouse anti-5HTT (mouse anti-serotonin transporter, monoclonal antibody 1:1,000, Millipore, Cat# MAB1564, RRID:AB_94220). Slices were incubated at 4°C overnight under agitation. After three washes in PBS, sections were incubated for 1.5 h in the dark with fluorescent secondary antibodies: goat anti-mouse IgG conjugated with Alexa Fluor™ 568 (1:1,000, Invitrogen, Cat# A11004, RRID:AB_143162) and goat anti-rabbit IgG conjugated with Alexa Fluor™ 488 (1:1,000, Invitrogen, Cat# A11008, RRID:AB_143165). In each immunofluorescence study, a negative control was performed omitting the primary antibody to ensure technique specificity (see **Supplementary Figure S2**). Sections were later counterstained with Hoechst 33342 (1:1,000, Sigma-Aldrich) to label nuclei, mounted on gelatin-coated slides, and coverslipped with 70% glycerol mounting medium.

Photographs were taken in an inverted Olympus IX83 microscope with several objectives (4×, 10×, 20×). For double immunofluorescence studies, an objective of 60× and an additional spinning disk unit (SDU) for better resolution were used to analyze two markers in a brain area and to show marker colocalization. Images were acquired using high-resolution digital monochromatic sCMOS *Orca* camera (Hamamatsu) and *CellSens Dimension CS-DI-V1* software.

Immunoperoxidase

Five coronal 50-μm-thick brain sections containing ACC and amygdala were randomly selected from five mice per group, each one from different litters. Slices were washed three times in PBS and immersed in a solution of 0.5% (v/v) H_2O_2 in PBS for 1 h at room temperature under agitation to inhibit endogen peroxidase. Sections were then washed three times in PBS and immersed in a solution of 3% (v/v) normal equine serum plus 0.5% (v/v) Triton X-100 in PBS for 1.5 h at room temperature under agitation to permeabilize and block unspecific sites. Sections were then incubated with the following primary antibodies diluted in a solution of 1% (v/v) normal equine serum and 0.3% (v/v) Triton X-100 in PBS: mouse anti-MAP2 (mouse anti-microtubule-associated protein type 2, monoclonal antibody, 1:1,000, Sigma-Aldrich, Cat# M4403, RRID:AB_477193) and mouse anti-NF200 (mouse anti-neurofilament 200 kDa, monoclonal antibody, 1:1,000, Sigma-Aldrich, Cat# N0142, RRID:AB_477257). Slices were incubated at 4°C overnight under agitation. After three washes in PBS, sections were incubated for 1.5 h with goat anti-mouse IgG biotin conjugated (whole molecule, polyclonal antibody, 1:1,000, Sigma-Aldrich Cat# B7264, RRID:AB_258607). After three washes in PBS, sections were incubated with extravidin peroxidase solution (1:500, Sigma-Aldrich, Cat# E2886) followed by two washes in PBS and two with acetate buffer (AB) 0.1 M pH 6. Slices were incubated with 0.035% (w/v) 3,3′ diaminobenzidine (Sigma Aldrich) and 4% (w/v) nickel ammonium sulfate in AB, added H_2O_2 to reveal color, then washed twice with AB, and finally washed with distilled water. Slices were mounted on gelatin-coated slides and coverslipped using Canada Synthetic Balm as mounting media.

Photographs were taken on a Zeiss Axiolab microscope with several objectives (2.5×, 10×, 20×). Images were acquired using CCD *Q-Color 3* camera (Olympus) and *QCapture 6.0* software.

Morphometric Digital Image Analysis

All measurements were made on the photomicrographs taken with the corresponding microscopes and analyzed by two blinded operators. ACC and amygdala were the two brain areas selected for morphometric studies, and all the measurements were made using ImageJ software (NIH[1]).

From immunostaining, the number of neuron nuclei per unit of area, the percentage of area covered by 5HTT, 5HT, NF200, and MAP2-positive fibers as well as the percentage of area covered by 5HT1AR and CB1R-positive

[1] http://imagej.net

immunostained structures were measured in 20× primary magnification images. The number of cells per unit of area was determined by quantification of Hoechst-positive nuclei at 20× primary magnification. The percentage of area covered by immunolabeled fibers or receptor was related to the total area of the corresponding microscopic field at 20× primary magnification.

To measure the level of dispersion of the directionality of NF200-positive fibers and MAP2-positive dendrite orientation, ACC photomicrographs for each marker were analyzed with ImageJ. The Directionality plugin was used following instructions on https://imagej.net/Directionality, which exploits the local gradient orientation method (Schindelin et al., 2012; Schneider et al., 2012) for this quantification. For more information about quantification with this plugin, see **Supplementary Material** Section 3.

To measure 5HT and 5HTT colocalization in ACC and amygdala, photomicrographs of double-immunostaining with both markers were analyzed with ImageJ, using the JACoP plugin. The threshold from which it was considered a positive mark was set for each marker and the Manders' overlap coefficient was calculated (Manders et al., 1992; Bolte and Cordelières, 2006).

Data Analysis

Statistical analysis was performed using GraphPad Prism v5.00 (GraphPad Software Inc.). In behavioral tests, an average for each litter from both experimental groups was calculated (see **Supplementary Table S1**). A statistical Student's *t*-test was performed to compare the means of the averages per litter of Control and PEE groups for all parameters. Model assumptions were verified in all cases.

Immunostaining quantifications (n = 3–5/treatment) were made from three slices per brain of each treatment and brain structure. Means and standard error of the mean (SEM) were obtained for all variables measured, the assumptions of normality and homoscedasticity were tested, and a two-tailed Student's *t*-test was performed to compare the two groups.

RESULTS

Physical Parameters of PEE Mice

Neither microcephaly nor any physical malformation was observed in PEE pups. There were neither litter size variations (Control 13.83 ± 0.4773 n = 6 vs. PEE 13.17 ± 0.7923 n = 6, Student's *t*-test, ns) nor weight deviations in P21 (Control 12.75 g ± 0.4787 n = 4 vs. PEE 12.50 g ± 0.2887 n = 4, Student's *t*-test, ns) and adulthood (Control 34.13 ± 0.6152 n = 32 vs. PEE 35.32 ± 0.4962 n = 28, Student's *t*-test, ns) between PEE and Control pups.

There were no differences observed between the behavior of C and E mothers regarding the maternal care parameters qualitatively assessed. Ethanol mothers' consumption during pregestational, gestational, and lactation periods is shown in **Supplementary Figure S1**. There were no differences between E and C mothers in weight gain and beverage intake during pregestational, gestational, and lactation periods (**Supplementary Figure S1**). E mothers yielded BEC values of 73.29 ± 8.69 mg/dl (n = 3) at the end of lactation, while female PEE pups yielded a BEC of 101.56 ± 5.21 mg/dl (n = 2) at P21.

Behavioral Studies

LDB

PEE mice showed significant differences with respect to Control ones, spending less time in the light compartment ($t_{(10)}$ = 2.414, p = 0.0364; **Figure 2A**), which indicates anxiety-like behavior. Although no significant differences were observed, it can be noticed that PEE males tend to spend more time in dark compartment compared to Controls (**Figure 2B**), which also suggests an increase in anxiety. In turn, the number of transitions between the two compartments did not vary (**Figure 2C**), indicating that exploration was not affected by perinatal EtOH exposure.

OF

PEE mice spent significantly less time in the central area ($t_{(10)}$ = 3.784, p = 0.0036; **Figure 3A**) and more time in the periphery ($t_{(10)}$ = 5.421, p = 0.0003; **Figure 3B**), and presented a tendency to exhibit shorter latency times than Control animals (**Figure 3C**). All these results are consistent with one another and indicate an anxiogenic effect of perinatal EtOH. The distance traveled (**Figure 3D**) and the number of rearings and thigmotaxis did not differ between groups (**Figure 3E**), which indicates unaltered horizontal and vertical locomotion in PEE animals and may reflect a certain specificity of the effect of EtOH on anxious behavior.

Morphometric Parameters of ACC and Amygdala

Neither the organization of the six cortical layers of the ACC nor its thickness showed differences between PEE and Control groups (**Supplementary Figure S4E**). In both PEE animals and Controls, a radial organization of the cells of the cingulate cortex was observed towards the cingulum (**Supplementary Figures S4A–D**).

As shown in the histological analyses of the basolateral area, the amygdala cytoarchitecture was also conserved in PEE animals regarding the appearance observed in Controls (**Supplementary Figures S5A–D**). The area occupied by the amygdala in brain slices at the same Bregma level did not differ between the two groups (**Supplementary Figure S5E**).

The axonal cytoskeleton, immunolabeled for NF200 protein in ACC, was altered in adult PEE males (**Figures 4A–D**). Control animals showed axonal fibers with a radial distribution in this structure, while PEE animals exhibited disorganized axonal distribution through the six typical layers of the cerebral cortex ($t_{(6)}$ = 4.054, p = 0.0067; **Figure 4F**) and smaller area covered ($t_{(6)}$ = 3.582, p = 0.0116; **Figure 4E**).

Immunostaining for MAP2 protein (**Figures 5A–D**), which allows the identification of neurons and dendritic prolongation, also showed ACC radial organization in Controls but not in PEE animals ($t_{(6)}$ = 3.612, p = 0.0112; **Figure 5F**), although the area covered by these fibers did not differ between groups (**Figure 5E**).

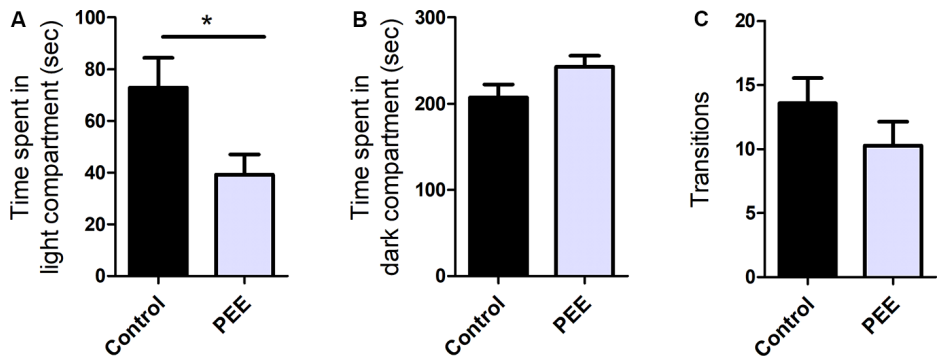

FIGURE 2 | Perinatal EtOH exposure increases anxiety responses in the light-dark box test (LDB) test. Time spent in light compartment (in seconds, **A**), time spent in dark compartment (in seconds, **B**), number of transitions between the two compartments **(C)**. Data expressed as the mean ± standard error of the mean (SEM); all parameters were analyzed by Student's *t*-test (*$p < 0.05$). Control $n = 6$; PEE $n = 6$, each data correspond to the average per litter.

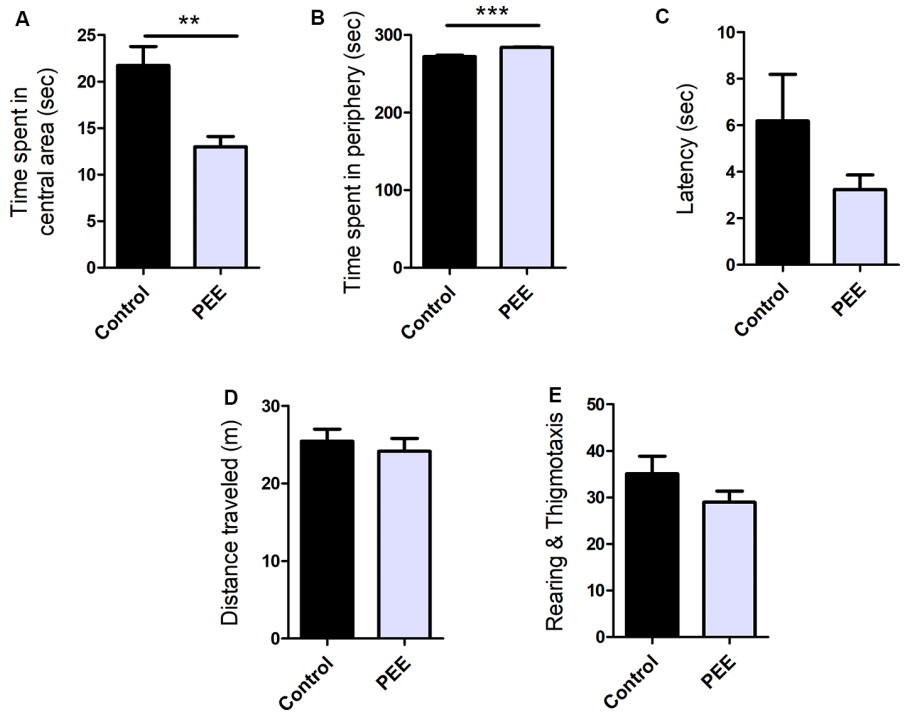

FIGURE 3 | Perinatal EtOH exposure increases anxiety responses in the open field test (OF) test. Time spent in central area (in seconds, **A**), time spent in peripheral area (in seconds, **B**), latency to leave the center (in seconds, **C**), total distance traveled (in meters, **D**), number of rearings and thigmotaxis events **(E)**. Data expressed as the mean ± SEM; all parameters were analyzed by Student's *t*-test (**$p < 0.01$, ***$p < 0.001$). Control $n = 6$; PEE $n = 6$, each data correspond to the average per litter.

Further histological analyses of the ACC (**Figures 6A,E**) regarding the cellularity and percentage of mature neurons (**Figures 6B,F**) showed no alterations in PEE animals (**Figures 6I,J**). In addition, the area covered by CB1R (**Figures 7C,G**) in this structure showed no significant differences between groups (**Figures 6C,G,K**).

At higher magnification, the expression of CB1R on the ACC is shown. CB1R is highly expressed around the mature neurons of the ACC in both Control and PEE brains (**Figures 6D,H**).

Studies conducted on the same parameters in amygdala (**Figures 7A,E**) revealed a conserved total cell number in PEE adults (**Figures 7I**) but a smaller percentage of mature neurons (**Figures 7B,F,J**) ($t_{(8)} = 5.353$, $p = 0.0007$) regarding Controls (**Figures 7I,J**). The area covered by CB1R (**Figures 7C,G**) in this structure was significantly smaller in PEE animals compared to Controls ($t_{(6)} = 8.081$, $p = 0.0002$; **Figures 7K**).

At higher magnification, the expression of CB1R on the amygdala is shown. CB1R is highly expressed around mature

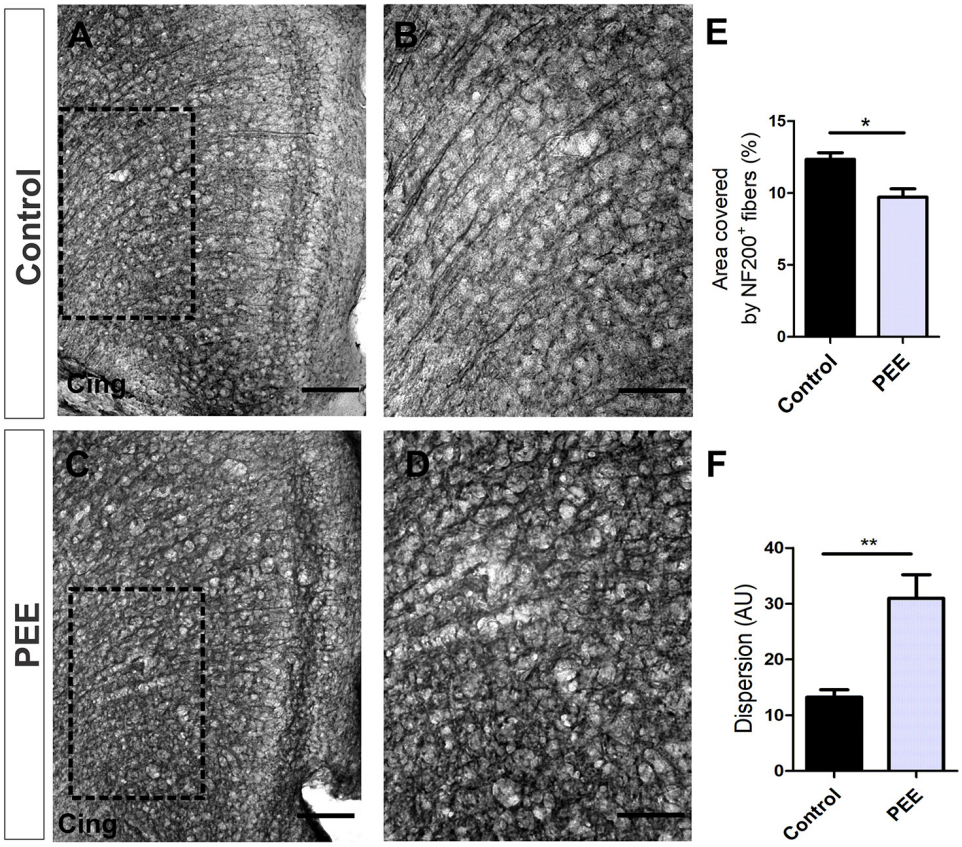

FIGURE 4 | The axonal cytoskeleton is altered in adult PEE adults. Optical photomicrographs of coronal sections of adult male mouse brains immunostained with NF200. Height of the anterior cingulate cortex (ACC) of a Control **(A)** and PEE brain **(C)** at low magnification. Height of the ACC of a Control **(B)** and PEE brain **(D)** at higher magnification. In **(A,C)** photomicrographs, the cingulum is indicated with the abbreviation cing. Area covered by NF200$^+$ fibers (%, **E**). Dispersion grade in the orientation of the NF200$^+$ fibers is expressed in arbitrary units (AU; **F**). Data expressed as the mean ± SEM (Control $n = 4$ each one from four different control litters, PEE $n = 4$ each one from four different ethanol PEE litters); all parameters were analyzed by Student's t-test (*$p < 0.05$, **$p < 0.01$). Scale bars: 100 μm **(A,C)**, 50 μm **(B,D)**.

amygdala neurons in Control brains, but in PEE ones, this expression is reduced (**Figures 7D,H**).

Regarding serotonergic neuromodulation, PEE animals showed alterations in serotonergic afferences (**Figures 8A–O** and **Figures 9A–O**) as evidenced by a decrease in the area covered by fibers immunostained with the serotonin transporter in both the ACC ($t_{(7)} = 5.564$, $p = 0.0008$; **Figure 8M**) and amygdala ($t_{(6)} = 2.861$, $p = 0.0287$; **Figure 9M**). These 5HTT-positive fibers were also 5HT-positive, as shown in merge images of **Figures 8F,L**, **9F,L**. The area covered by 5HT$^+$ immunostaining was lower in PEE animals in both ACC ($t_{(7)} = 2.518$, $p = 0.0399$; **Figure 8N**) and amygdala ($t_{(6)} = 6.613$, $p = 0.0006$; **Figure 9N**). Taking both results, we can conclude that the serotonergic innervation was lower in PEE animals.

The Manders' overlap coefficient mean value was near 0.8 in all cases and did not differ between the two groups (**Figures 8O**, **9O**), which indicates that there is an approximately 80% of superposition of 5HTT$^+$ and 5HT$^+$ immunofluorescent structures and confirms that 5HTT$^+$ fibers contain the neurotransmitter 5HT.

In addition, PEE males showed alterations in 5HT1A receptor levels regarding Controls (**Figures 10A–I** and **Figures 11A–I**) with a decrease in the ACC ($t_{(6)} = 3.136$, $p = 0.0202$; **Figure 10I**) and an increase in the amygdala ($t_{(5)} = 2.943$, $p = 0.0321$; **Figure 11I**).

At higher magnification, the expression of the 5HT1A receptor around the mature neurons of the ACC can be observed, which is lower in the ACC of PEE animals (**Figures 10D,H**). On the contrary, in the amygdala, the expression of this receptor around the mature neurons in the PEE animals is higher than the Controls (**Figures 11D,H**).

DISCUSSION

The experience and behavior that parents have had prior to conception can affect future offspring, as behavioral patterns such as diet (Öst et al., 2014), exercise (Denham, 2018) or drug exposure (Minnes et al., 2014) may generate epigenetic marks in individuals, such as DNA methylation. In this way, the offspring inherit not only the genes of the parents but also their previous

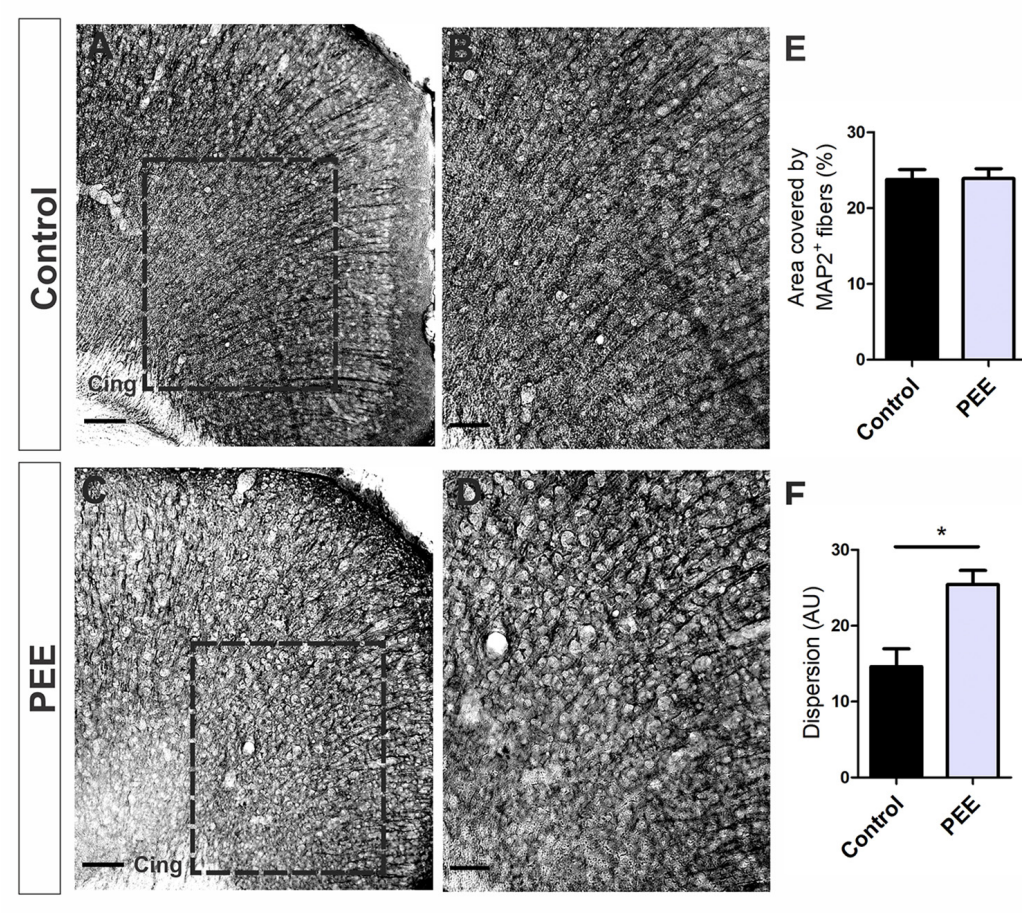

FIGURE 5 | The dendritic cytoskeleton is altered in PEE adults. Optical photomicrographs of coronal sections of adult male mouse brains immunostained with MAP2. Height of the ACC of a Control **(A)** and PEE brain **(C)** at low magnification. Height of the ACC of a Control **(B)** and PEE brain **(D)** at higher magnification. Dispersion grade in the orientation of $MAP2^+$ processes is expressed in arbitrary units (AU, **F**). In **(A,C)** photomicrographs, the cingulum is indicated with the abbreviation cing. Area covered by $MAP2^+$ fibers (%, **E**). Data expressed as the mean ± SEM (Control $n = 4$ each one from four different control litters, PEE $n = 4$ each one from four different ethanol PEE litters); all parameters were analyzed by Student's *t*-test ($^*p < 0.05$). Scale bars: 100 μm **(A,C)**, 50 μm **(B,D)**.

experience translated into epigenetic marks. In the current study, the EtOH exposure animal model contemplates not only the direct impact of this drug on the gestation and lactation but also the effects of pregestational exposure, i.e., EtOH consumption by the dam prior to pairing. Our mouse model thus intends to reproduce the pattern of EtOH consumption of an alcoholic mother, taking into account experiences prior to the conception of the offspring.

One of the problems associated to the administration of EtOH into the beverage is that rodents tend to dislike it. Reports have shown that Wistar rats refuse to drink EtOH 10% v/v but are capable of drinking a solution of EtOH 6% v/v in water for 4 weeks, with no symptoms of toxic effect in hepatic tissue or alterations in their ability to mate, pregnancy parameters, lactation, or pup care, and yield moderate to low BEC values in both dams and pups (Evrard et al., 2003). In contrast, intraperitoneal administration through an injection of 3.5 g/kg/day to pregnant Wistar rats from G10 to G18 has rendered higher BEC and some teratogenic consequences (Aronne et al., 2008). In addition, pregnant Long Evans rats intraperitoneally injected with EtOH 2.9 g/kg on G15 and EtOH 1.45 g/kg 2 h later have shown BEC values of 287 ± 3.5 mg/dl (Mooney and Varlinskaya, 2011). Furthermore, pregnant CD1 females administered EtOH 25% v/v in the beverage have rendered pregnancy BEC values of 100–140 mg/dl (Kozanian et al., 2018).

The protocol used in this work shows that the BEC reached by E mothers was slightly higher than the legal limit for driving in Argentina (50 mg/dl) and below the levels of overt drunkenness in humans (200 mg/dl). In this way, considering that consequences of prenatal EtOH exposure depend on the dose, period, and duration of EtOH exposure (Petrelli et al., 2018), the current work contemplates the consequences that moderate and prolonged maternal consumption of EtOH may have on offspring and might be thus thought to mimic cases of FASD.

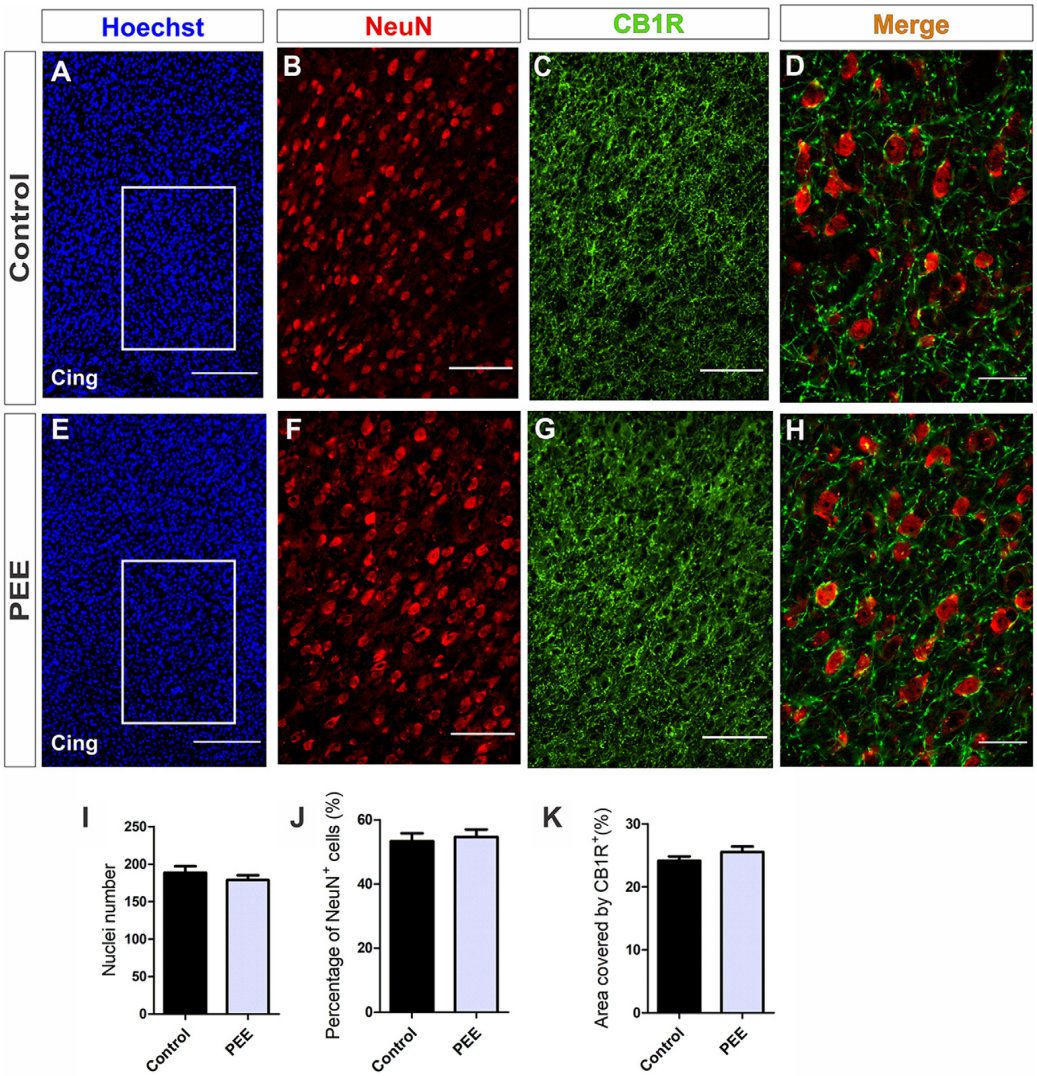

FIGURE 6 | The population of mature neurons and the area covered by CB1 receptor (CB1R) are conserved in the anterior cingulate cortex (ACC) of PEE adults. Images of coronal sections of adult male mouse brains with Hoechst staining (blue) and immunofluorescence for NeuN (red) and CB1R (green) taken on an inverted microscope with spinning disk unit (SDU). Sections at the level of the ACC of a Control **(A)** and PEE brain **(E)** at low magnification. Sections at the level of the ACC of a Control **(B,C)** and PEE brain **(F,G)** at higher magnification. The merge of NeuN and CB1R immunofluorescence is shown at 60× magnification **(D,H)**. In **(A,D)** photomicrographs, the cingulum is indicated with the abbreviation cing. Nuclei number **(I)**, percentage of $NeuN^+$ cells (%, **J**), area covered by CB1R (%, **K**). Data expressed as the mean ± SEM (Control $n = 5$ each one from five different control litters, PEE $n = 5$ each one from five different ethanol PEE litters); all parameters were analyzed by Student's t-test. Scale bars: 200 μm **(A,E)**, 75 μm **(B,C,F,G)**, 25 μm **(D,H)**.

Previous studies by our group using administration of EtOH 6% v/v to Wistar rats through a liquid diet before and during gestation have shown dam BEC of 89.34 ± 6.42 mg/dl, as well as alterations in fetal brain morphology that affect the development of radial glia and hence cause a delay in migration. This could induce a disruption in the structure and function of major CNS laminated structures such as the cerebral cortex (Aronne et al., 2011). On the other hand, adolescent rats perinatally exposed to EtOH at this concentration show a higher preference for EtOH (more significant in females than males) and behavioral alterations (Aronne et al., 2013). In other words, prenatal or perinatal EtOH exposure in rodents produces changes in behavior that have been extensively studied from early postnatal days until early adulthood and has an impact on CNS structures that could be related to these alterations. Similar protocols of exposure to EtOH drinking have also been used in different mouse strains, some of them even involving a high EtOH concentration as the only beverage (Kleiber et al., 2011; El Shawa et al., 2013; Vega et al., 2013; Pérez-Tito et al., 2014; Abbott et al., 2016). At the moment, however, no conclusive results have been obtained regarding the behavior of adult male mice perinatally exposed to low/moderate concentrations of EtOH.

Anxiety is defined as a negative emotional state associated with the perception of potential or ambiguous

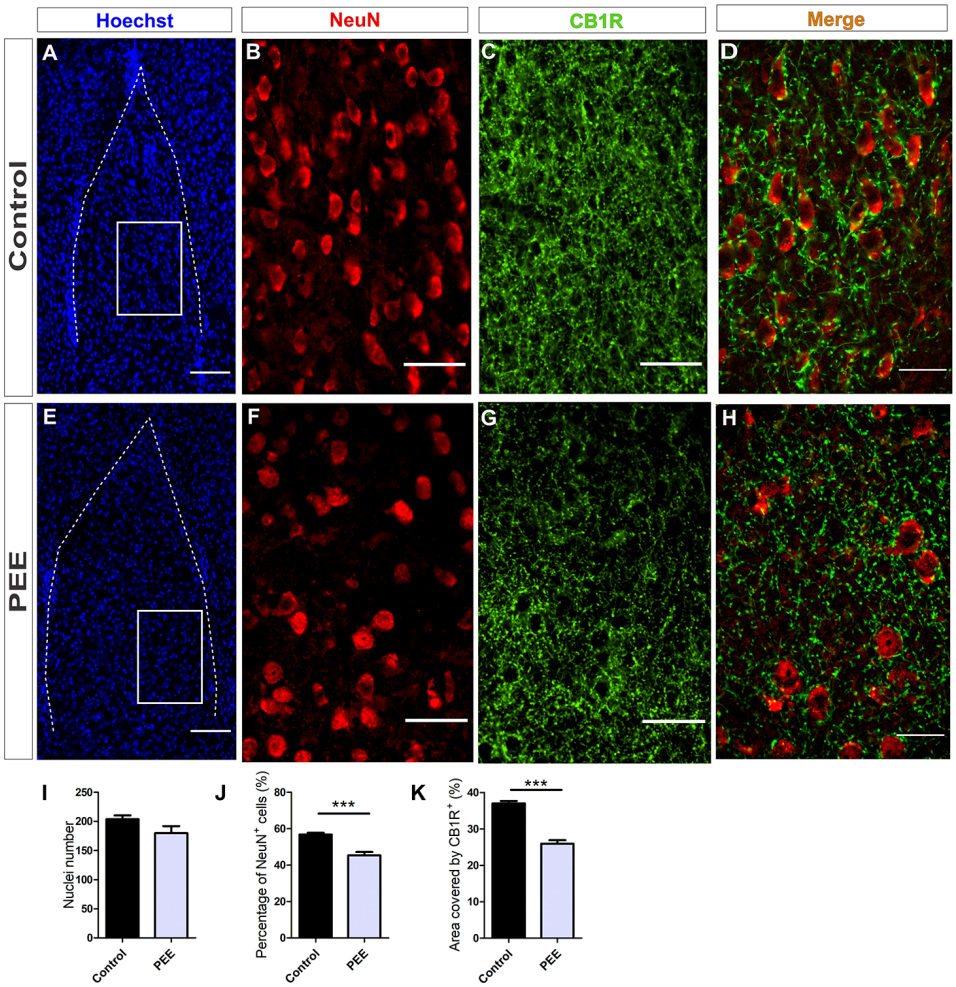

FIGURE 7 | The population of mature neurons and the area covered by CB1R are smaller in the amygdala of PEE adults. Images of coronal sections of adult male mouse brains with Hoechst staining (blue) and immunofluorescence for NeuN (red) and CB1R (green) taken on an inverted microscope with SDU. Sections at the level of the amygdala of a Control **(A)** and PEE brain **(E)** at low magnification. Sections at the level of the amygdala of a Control **(B,C)** and PEE brain **(F,G)** at higher magnification. The merge of NeuN and CB1R immunofluorescence is shown at 60× magnification **(D,H)**. Nuclei number **(I)**, percentage of $NeuN^+$ cells (%, **J**), area covered by CB1R (%, **K**). Data expressed as the mean ± SEM (Control $n = 5$ each one from five different control litters, PEE $n = 5$ each one from five different ethanol PEE litters); all parameters were analyzed by Student's *t*-test (***$p < 0.001$). Scale bars: 100 μm **(A,E)**, 50 μm **(B,C,F,G)**, and 25 μm **(D,H)**.

threat. No unequivocal measures of anxiety have been yet established for rats and mice; however, and even when they may render differences between strains or face methodological criticism, the LDB and OF tests are generally accepted as a measurement of rodent anxious behavior, as they may assess fear-induced escape/avoidance or spontaneous natural preference for enclosed or unlit spaces (Ennaceur, 2014).

Previous studies in adult rodents prenatally exposed to EtOH showed similar results to those observed in this work, even using treatments applied in different time windows, with higher concentrations of EtOH and supplied through different routes (Hellemans et al., 2008; Kleiber et al., 2011; Cullen et al., 2013; Wieczorek et al., 2015). In addition, behavioral studies in rats prenatally exposed to EtOH between G17 and G20 have revealed an anxious phenotype in childhood and adolescence (Wille-Bille et al., 2018). In contrast, other authors have reported a decrease in anxiety-like behavior in adult and adolescent rodents prenatally exposed to EtOH, even using similar treatments and the same behavioral tests used in the current work (Osborn et al., 1998; Allan et al., 2003; Carneiro et al., 2005; Ohta et al., 2010; Diaz et al., 2016). Other groups have demonstrated that CD1 mice exposed to EtOH 25% v/v during gestation show an anxious phenotype at P20 and P50 (El Shawa et al., 2013; Abbott et al., 2016). In this work, adult male PEE mice of the CD1 strain exhibited an anxious phenotype that was consistent throughout the behavioral tests used. This phenotype is in agreement with that observed in humans, with studies showing that children, adolescents, and even adults prenatally exposed to EtOH present frequent psychiatric disorders such as anxiety (Famy et al., 1998; O'Connor and Paley, 2009; Popova et al., 2016; Weyrauch et al., 2017).

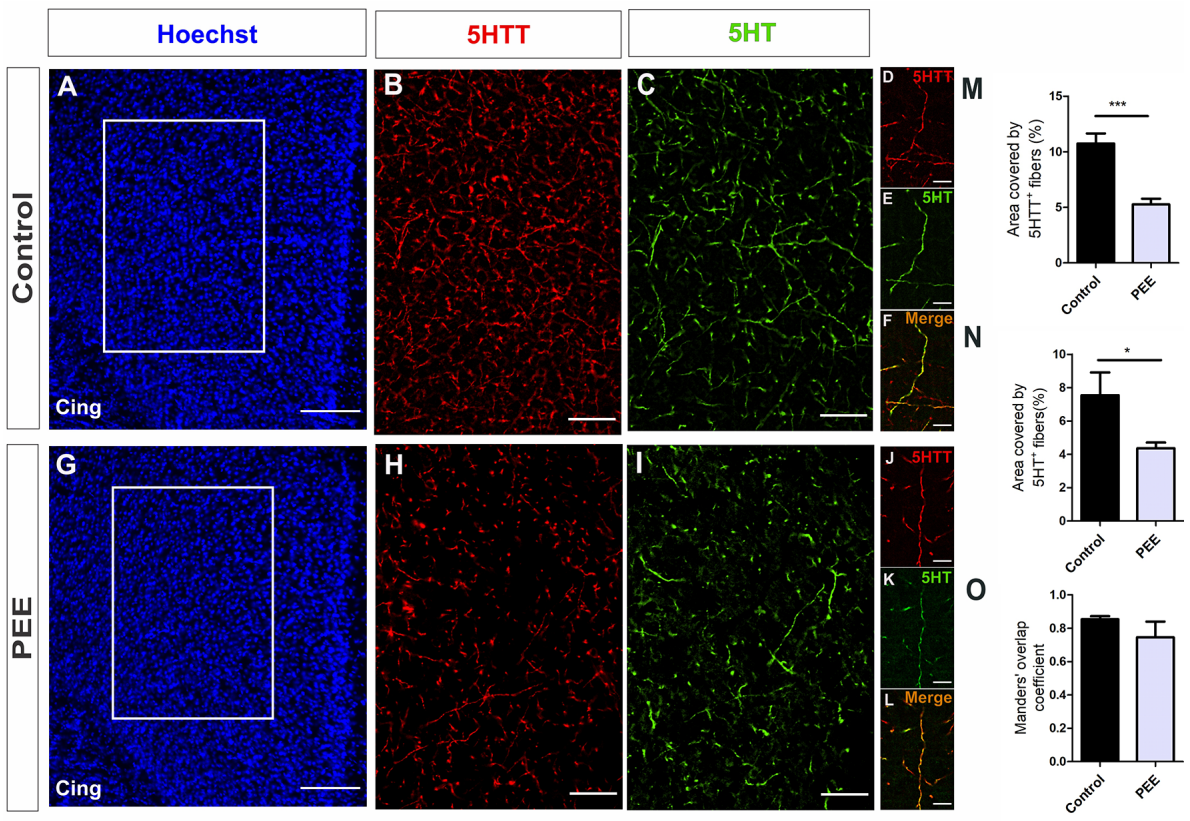

FIGURE 8 | Serotoninergic innervation is altered in the ACC of adult PEE mice. Images of coronal sections of adult male mouse brains with Hoechst staining **(A,G)** and immunostained for 5HTT **(B,D,H,J)** and 5HT **(C,E,I,K)**. Sections at the level of the ACC of a Control **(A–F)** and PEE brain **(G–L)**. Merge images **(F,L)** show that fibers containing 5HTT also contain 5HT. In **(A,G)** photomicrographs, the cingulum is indicated with the abbreviation cing. Area covered by $5HTT^+$ fibers (%, **M**), area covered by 5HT+ (%, **N**), and Manders' overlap coefficient **(O)**. Data expressed as the mean ± SEM (Control $n = 4$ each one from four different control litters, PEE $n = 5$ each one from five different PEE litters); all parameters were analyzed by Student's t-test ($^*p < 0.05$, $^{***}p < 0.001$). Scale bar: 150 μm **(A,G)**, 50 μm **(B,C,H,I)**, and 10 μm **(D–F,J–L)**.

The formation of the complex architecture of the mammalian cerebral cortex requires orchestrated events including neural stem cell proliferation, migration, and neuronal differentiation. Successful neural migration involves three basic steps: initial departure of neuroblasts from the ventricular zone, migration to the cortical plate, and final settlement at their intrinsic laminar positions (Pang et al., 2008). Cortical connections formed during gestation and infancy are modified through synaptic pruning and cellular apoptosis. We have shown in previous work that the cerebral cortex of fetuses exposed to EtOH has a delay in neuroblast migration that produces alterations in lamination (Aronne et al., 2011) and that adult offspring prenatally exposed to EtOH have a thinner cerebral cortex, also with alterations in lamination (Aronne et al., 2013). These results confirm that exposure to EtOH during brain development produces morphological changes that persist into adulthood even in the absence of EtOH consumption.

Similar results, including low body and brain weights as well as lower cerebral cortex thickness, have been observed in infant and adult CD1 mice prenatally exposed to EtOH 25% v/v during gestation (El Shawa et al., 2013; Abbott et al., 2016). In addition, C57Bl/6 mice exposed to a liquid diet of EtOH 10% v/v from 15 days before pregnancy up to P4 revealed a reduction in olfactory bulb, hippocampus granule cell layer of the dentate gyrus, and fourth ventricle volume in adulthood, but larger amygdala volume (Akers et al., 2011).

Moreover, previous studies in which female mice were treated with EtOH 10% v/v prior to conception and during gestation and lactation have evidenced an anxiety-like behavior in youth offspring (Kleiber et al., 2011). On the other hand, Pascual et al. (2017) observe an anxiety-like behavior in adult PEE offspring exposing the dams to EtOH 2 months before conception until the end of lactation. In this work, authors also observed an increase in markers associated with inflammation processes in the brain that could be related with the neurodevelopmental defects registered (Pascual et al., 2017).

In turn, the brain region evaluated in this work is associated with cognitive processes (Kim et al., 2014; Meechan et al., 2015) and complex behavior such as response to fear and anxiety (Jhang et al., 2018; Sah et al., 2019). In particular, studies focused on areas of the limbic system like the cingulate cortex and amygdala,

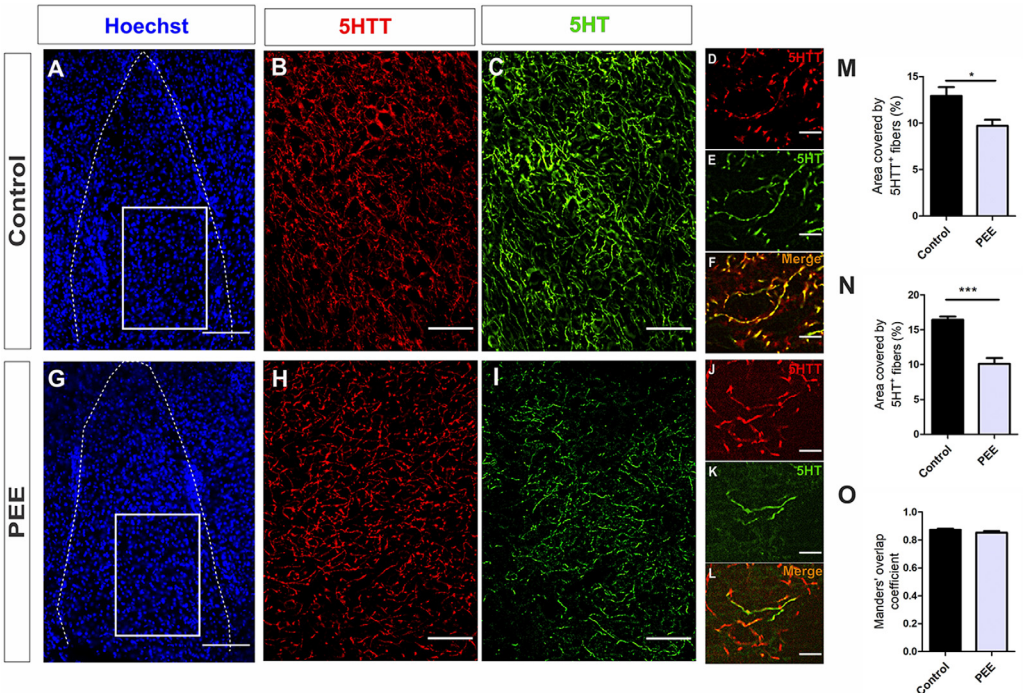

FIGURE 9 | Serotoninergic innervation is altered in the amygdala of adult PEE mice. Images of coronal sections of adult male mouse brains with Hoechst staining **(A,G)** and immunostained for 5HTT **(B,D,H,J)** and 5HT **(C,E,I,K)**. Sections at the level of the amygdala of a Control **(A–F)** and PEE brain **(G–L)**. Merge images **(F,L)** show that fibers containing 5HTT also contain 5HT. In **(A,G)** photomicrographs, the cingulum is indicated with the abbreviation cing. Area covered by $5HTT^+$ fibers (%, **M**), area covered by 5HT+ (%, **N**), and Manders' overlap coefficient **(O)**. Data expressed as the mean ± SEM (Control $n = 4$ each one from four different control litters, PEE $n = 4$ each one from four different PEE litters); all parameters were analyzed by Student's t-test ($^*p < 0.05$, $^{***}p < 0.001$). Scale bar: 150 μm **(A,G)**, 50 μm **(B,C,H,I)**, and 10 μm **(D–F,J–L)**.

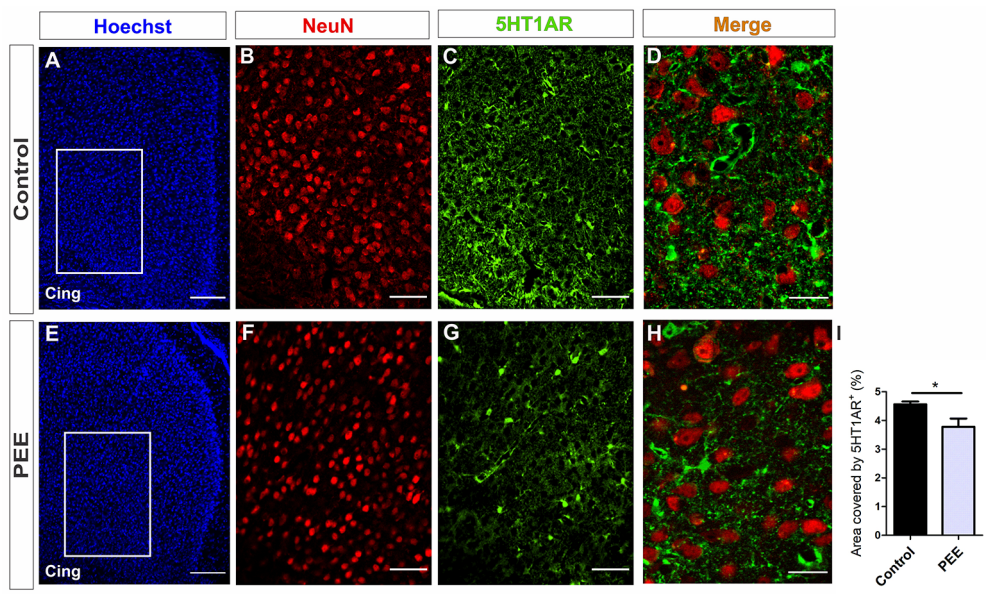

FIGURE 10 | 5HT1A receptor expression is altered in the ACC of PEE adults. Images of coronal sections of adult male mouse brains with Hoechst staining **(A,E)**, immunofluorescence for NeuN **(B,F)**, 5HT1A receptor (5HT1AR; **C,G)**, and merge **(D,H)** taken on an inverted microscope with a SDU. Sections at the level of the ACC of a Control **(A–D)** and PEE brain **(E–H)**. In A and G photomicrographs, the cingulum is indicated with the abbreviation cing. Area covered by $5HT1AR^+$ measured in the fields delimited by boxes (%, **I**). Data expressed as the mean ± SEM (Control $n = 5$ each one from five different control litters, PEE $n = 3$ each one from three different ethanol PEE litters); all parameters were analyzed by Student's t-test ($^*p < 0.05$). Scale bar: 150 μm **(A,E)**, 50 μm **(B,C,F,G)**, and 20 μm **(D,H)**.

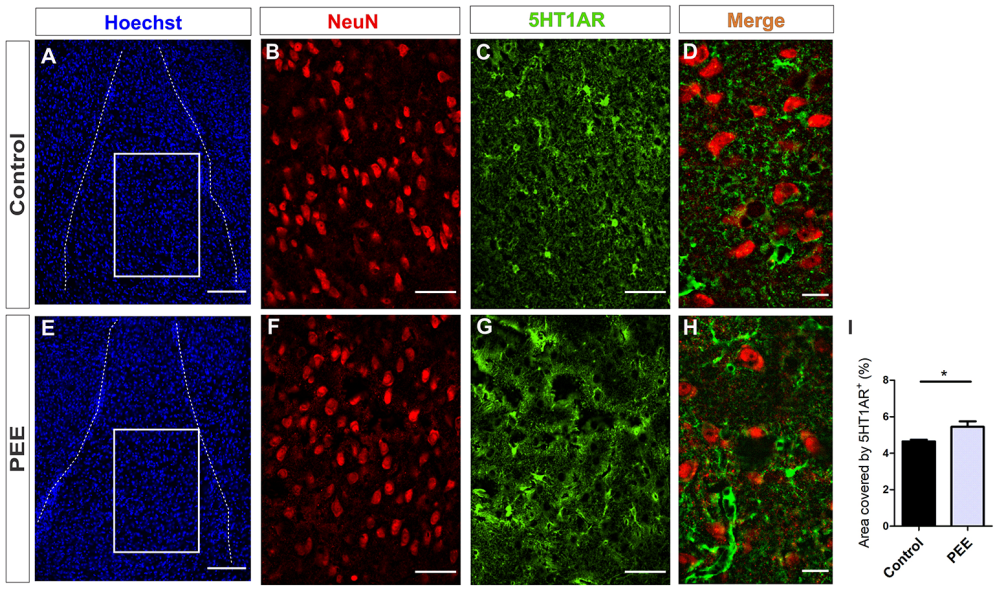

FIGURE 11 | 5HT1A receptor expression is altered in the amygdala of PEE adults. Images of coronal sections of adult male mouse brains with Hoechst staining **(A,E)**, immunofluorescence for NeuN **(B,F)**, 5HT1AR **(C,G)**, and merge **(D,H)** taken on an inverted microscope with a SDU. Sections at the level of the amygdala of a Control **(A–D)** and PEE brain **(E–H)**. Area covered by 5HT1AR$^+$ measured in the fields delimited by boxes (%, **I**). Data expressed as the mean ± SEM (Control $n = 4$ each one from four different control litters, PEE $n = 3$ each one from three different ethanol PEE litters); all parameters were analyzed by Student's *t*-test ($^*p < 0.05$). Scale bar: 150 μm **(A,E)**, 50 μm **(B,C,F,G)**, and 15 μm **(D,H)**.

whose cytoarchitecture and state of synaptic connections may be linked to alterations in functionality and, ultimately, in the behavioral aspects they regulate. Therefore, the reduction observed in the population of mature neurons in the amygdala of PEE adult mice could be related to the anxious phenotype recorded in them. Similar results on the correspondence between morphology of the amygdala and behavior in adult CD1 mice prenatally exposed to EtOH have been obtained by other authors (Kozanian et al., 2018). Moreover, these results could be linked to clinical evidence showing that patients with autism have an increase in anxiety and a lower number of neurons in this structure (Schumann and Amaral, 2006).

NF200, a dynamic element of the neuronal cytoskeleton, determines axonal caliber and is necessary for axonal growth and guidance on their way to the synaptic target, as well as for neuronal shaping (Hoffman et al., 1987). We have observed a decrease in NF200 expression in the cingulate cortex of PEE adult mice that could be related to its functionality, either by an alteration in efferences, causing changes in the behavior it controls, or by an alteration in afferences, altering its regulation and, consequently, events downstream.

It is known that in mice prenatally exposed to EtOH, mesencephalic serotonin nuclei have a lower number of serotonergic neurons at P45 (Sari and Zhou, 2004) and a lower content of serotonin in the whole adult brain (Krsiak et al., 1977). In our work, both 5HTT and 5HT immunofluorescence were used to evaluate serotonergic innervation in the ACC and amygdala, showing a significant decrease in the area covered by these fibers in both brain areas in PEE adults. This result implies an alteration in innervation and serotonergic control in these areas as a consequence of EtOH exposure during early brain development and is consistent with other reports showing alterations in 5HTT and 5HT levels in PEE offspring (Zafar et al., 2000; Ramos et al., 2002; Evrard et al., 2003). A decrease in 5HTT and 5HT may indicate a reduction in serotonergic innervation due to altered development in 5HT fibers as a result of PEE. Since the serotonergic system is neuromodulatory, this decrease may imply alterations in the regulation of these areas of the limbic system. Also, a decrease was observed in 5HT1AR expression in ACC, which, together with the decrease in 5HTT levels in this area, could indicate a deficit in serotonergic modulation as a consequence of PEE. 5HT1AR acts during early postnatal development to establish normal anxiety-like behavior in adults (Gross et al., 2002). Given this evidence, it might be speculated that a decrease in the levels of 5HTT, 5HT, and 5HT1AR in the ACC due to exposure to EtOH during fetal and early postnatal development is related to the anxiety-like behavior expressed in adulthood. In amygdala, however, a reduction in serotonergic innervation was accomplished through an increase in the expression of the 5HT1AR, suggesting that this brain structure has a compensatory response to the low level of 5HT in adult PEE.

The endocannabinoid system constitutes another neuromodulator and is associated to the regulation of anxious responses (Navarro et al., 1993; Rodríguez de Fonseca et al., 1997), with some reports specifically linking the basolateral amygdala with this type of behavior (Delgado et al., 2006).

It is well known that glutamatergic projections toward the ventral hippocampus give rise to anxious responses and that the inactivation of the amygdala blocks anxious behavior (Janak and Tye, 2015). In addition, previous studies have shown that CB1R in the basolateral amygdala is mainly located in the synaptic terminations of the GABAergic type (Katona et al., 2001). The cannabinoid system has a biphasic role in the control of anxiety, being located in both glutamatergic and GABAergic terminals, which exert their effects on anxiety in opposite ways (Millan, 2003). Therefore, this system may be thought to function as a "buffer," regulating the release of these two neurotransmitters in relation to alterations in serotonergic modulation also recorded in this area. Finally, in the current work, the expression of CB1R was found to decrease in the basolateral amygdala in PEE animals, with no changes in the ACC. Therefore, the cannabinoid system may regulate the functionality of the amygdala, a key structure of the limbic system due to its relationship with other areas, even with the cingulate cortex.

Some authors have suggested a possible crosstalk between the serotoninergic and endocannabinoid systems, demonstrating the presence of CB1 receptors in serotoninergic neurons (Lau and Schloss, 2008). A colocalization of CB1 receptor in serotoninergic fibers has even been demonstrated in the amygdala (Ashton et al., 2006; Häring et al., 2007). Taking into account the results presented in this work, it could be speculated that, in part, the decrease in CB1 levels in the amygdala of PEE animals could lead to alterations in the serotoninergic neuromodulation of this structure, which could have an impact on anxious behavior.

CONCLUSION

Exposure to low/moderate concentrations of EtOH from conception to childhood produces morphological changes in the brain that can be detected in adulthood even with no further EtOH consumption. In sum, some of the morphological alterations produced by EtOH are never reversed and remain in areas of the limbic system related to emotion where two of the main neuromodulatory systems, serotonergic and cannabinoid, also suffer alterations that might account for later anxious-like behavior.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by CICUAL, Facultad de Medicina, Universidad de Buenos Aires, Res. 2375/2017.

AUTHOR CONTRIBUTIONS

CM conducted all the steps in the experimental procedures (EtOH administration, control of gestation and lactation, behavioral tests, and morphological studies), data processing, statistical analysis, and wrote an initial draft of the article. NV participated with CM in animal fixations, immunostaining, and photographs. DS designed and analyzed with CM the behavioral tests. AB designed the experimental model, supervised the course of experiments, and wrote the final version of the article. All authors revised the final version of the article.

FUNDING

This work was supported by grants from Universidad de Buenos Aires (UBACYT 20020170100371BA) and FONCYT (PICT2017-0610), Argentina.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00092/full#supplementary-material.

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 29 June 2020
doi: 10.3389/fnbeh.2020.00082

Lingering Effects of Prenatal Alcohol Exposure on Basal and Ethanol-Evoked Expression of Inflammatory-Related Genes in the CNS of Adolescent and Adult Rats

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Edited by:
Angelina Pilatti, Consejo Nacional de Investigaciones Cientàficas y Técnicas (CONICET), Argentina

Reviewed by:
Roberto Sebastián Miranda-Morales, CCT CONICET Córdoba, Argentina
Rosana Camarini, University of São Paulo, Brazil

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Specialty section:
This article was submitted to Motivation and Reward, a section of the journal Frontiers in Behavioral Neuroscience

Received: *27 November 2019*
Accepted: *30 April 2020*
Published: *29 June 2020*

Citation:
Doremus-Fitzwater TL, Youngentob SL, Youngentob L, Gano A, Vore AS and Deak T (2020) Lingering Effects of Prenatal Alcohol Exposure on Basal and Ethanol-Evoked Expression of Inflammatory-Related Genes in the CNS of Adolescent and Adult Rats. Front. Behav. Neurosci. 14:82. doi: 10.3389/fnbeh.2020.00082

Emerging data suggest that alcohol's effects on central inflammatory factors are not uniform across the lifespan. In particular, prenatal alcohol exposure (PAE) significantly alters steady-state levels of neuroimmune factors, as well as subsequent reactivity to later immune challenge. Thus, the current experiment investigated developmental sensitivities to, and long-lasting consequences of, PAE on ethanol-evoked cytokine expression in male and female adolescent and adult rats. Pregnant dams received either an *ad libitum* ethanol liquid diet (2.2% GD 6–8; 4.5% GD 9–10; 6.7% GD11–20; 35% daily calories from ethanol) or free-choice access to a control liquid diet and water. At birth, offspring were fostered to dams given free-choice access to the control liquid diet. Pups then matured until mid-adolescence [postnatal day (PD) 35] or adulthood (PD90), at which time they were challenged with either a binge-like dose of ethanol (4 g/kg; intragastrically) or tap water. During intoxication (3 h post-ethanol challenge), brains and blood were collected for assessment of neuroimmune gene expression (reverse transcription-polymerase chain reaction; RT-PCR) in the hippocampus, amygdala, and PVN, as well as for blood ethanol concentrations (BEC) and plasma corticosterone levels. Results revealed that rats challenged with ethanol at either PD35 or PD90 generally exhibited a characteristic cytokine signature of acute intoxication that we have previously reported: increased *Il*-6 and *IkB*α expression, with decreased *Il-1*β and *Tnf*α gene expression. With a few exceptions, this pattern of gene changes was observed in all three structures examined, at both ages of postnatal ethanol challenge, and in both sexes. While few significant effects of PAE were observed for ethanol-induced alterations in cytokine expression, there was a consistent (but nonsignificant) trend for PAE to potentiate the expression of *Il*-6 and *IkB*α in all groups except adult females. Although these data suggest that later-life ethanol challenge was a far greater driver of inflammatory signaling than PAE, the current results demonstrate PAE resulted in subtle long-term alterations in the expression of many key neuroinflammatory factors associated with NF-κB signaling.

Such long-lasting impacts of PAE that may engender vulnerability to later environmental events triggering neuroinflammatory processes, such as chronic ethanol exposure or stress, could contribute to heightened vulnerability for PAE-related alterations and deficits.

Keywords: rat, ethanol, prenatal, neuroimmune, adolescent, cytokine, hippocampus, amygdala

INTRODUCTION

In utero exposure to alcohol produces a multitude of neurobehavioral deficits that exist on a continuum from mild to severe. Although the most severe deficits were first identified as fetal alcohol syndrome (FAS; Jones and Smith, 1973), the effects of prenatal alcohol exposure are now diagnosed as part of a spectrum of possible disorders, termed "fetal alcohol spectrum disorders" (FASD; Mattson et al., 2019; Welch-Carre, 2005). Despite the known risks and consequences of gestational alcohol exposure, a recent report indicated that approximately one in nine women in the US reported drinking at least one alcoholic drink during the past 30 days of their pregnancy, with about one-third of these women reporting binge-level drinking (Denny et al., 2019). Experts believe that, when considering the full range of FASDs, diagnosable rates of FASD are approximately 1% to 5% of the population among school-aged children (CDC, 2019[1]). This comes at a high societal cost, as a 2004 report estimated that the lifetime adjusted cost of caring for a single individual with FAS was approximately $2 million (Lupton et al., 2004).

Many significant neurobehavioral and cognitive effects of FASD have been identified, ranging from intellectual and learning disabilities to attentional deficits and impaired impulse control, and even greater alcohol intake later in life (Baer et al., 2003; Guerri et al., 2009; Mattson et al., 2019). In animal models of pre-and/or post-natal ethanol exposure, results are parallel to those seen in humans (Drew and Kane, 2014). In addition to potentiated ethanol consumption later in ontogeny (Chang et al., 2015; Fabio et al., 2015; Pueta et al., 2008; Youngentob and Glendinning, 2009), rodent offspring exposed to alcohol *in utero* also exhibit increased impulsivity, hyperactivity, attentional impairments, memory deficits, reduced behavioral flexibility, and social deficits (Brys et al., 2014; Juárez and Guerrero-Álvarez, 2015; Idrus et al., 2013; Kelly et al., 2000; Thomas et al., 2010; Waddell and Mooney, 2017). Furthermore, a wide range of structural and functional alterations in the brain have been observed in both human FAS/FASD cases and animal models of prenatal alcohol exposure. For example, a combination of post-mortem and *in vivo* imaging studies has demonstrated many structural abnormalities and functional alterations in the brains of individuals with FASD (Drew and Kane, 2014; Guerri et al., 2009). Animal models have reported parallel brain defects following pre-/early-postnatal alcohol exposure, including reduced cortical, hippocampal, and cerebellar volumes (Kane et al., 2014), as well as abnormalities in the corpus callosum, fiber tracts, and basal ganglia (Drew and Kane, 2014). These studies have demonstrated that the hippocampus and cerebellum are particularly vulnerable to prenatal alcohol exposure (PAE) effects.

Identification of the mechanisms by which PAE negatively influences normative brain development has been the focus of intensive investigation. More recently, ethanol-induced alterations in immune system function from PAE have emerged possible contributors to FASD-related abnormalities in both brain and behavior. A complicating factor in determining the influence of PAE on neuroimmune function across prenatal, adolescent, and adult stages is that the immune system itself is: (a) continually developing, yet not necessarily with a linear accretion of immunocompetence as a function of chronological age (Maggini et al., 2018); (b) sculpting neural circuits and other physiological differences, processes that can be interrupted by developmentally-timed insults (Lenz and Nelson, 2018); and (c) repeatedly responding to changes in the microbiota and external pathogens, contributing to individual differences in steady-state host defense in adulthood (Alpert et al., 2019). Indeed, studies examining the consequences of altering neuroimmune factors have revealed a wide variety of functional consequences (Yirmiya and Goshen, 2011), including abnormalities in neurogenesis, synaptogenesis, synaptic pruning, and myelination (Cai et al., 2000; Deverman and Patterson, 2009; Drew and Kane, 2014; Schwarz and Bilbo, 2012). There is a growing body of research indicating that PAE may cause lasting changes in immune system function and also engender vulnerability to later life immune, stress, or alcohol exposures) Noor and Milligan, 2018). Following PAE, offspring reportedly exhibit changes in steady-state levels of neuroimmune factors throughout ontogeny. For example, adult mice whose mothers drank ethanol during gestation and/or lactation revealed robust increases in toll-like receptor (TLR)-4, TLR-2, NF-kB-p65, and Interleukin (Il)-1β expression levels in the prefrontal cortex (PFC) and hippocampus when compared to offspring from mothers that did not consume ethanol (Cantacorps et al., 2017). Similarly, a more moderate and long-term PAE paradigm revealed lasting elevations in neuroimmune reporters in mice that were PAE offspring, which were observed from the embryonic period to the preweaning period, and then into adulthood (Pascual et al., 2017). Another study examining the effects of PAE in female rats reported significant increases in expression of Il-1β, Il-2, Il-4, Il-5, tumor necrosis factor (TNF)-α and interferon (IFN)-γ in the PFC (Bodnar et al., 2016).

While experiments such as these provide critical evidence that prenatal alcohol significantly alters the neuroimmune milieu in exposed offspring, many of these studies have focused on the effects of PAE very early in life or during adulthood (Drew et al., 2015). Less is known about the effects of PAE on neuroimmune function during the critical developmental

[1]statistics retrieved from: https://www.cdc.gov/ncbddd/fasd/data.html#ref

period of adolescence. In one study that examined the effects of PAE from gestational day (GD) 7–9, significant upregulation of Il-1β, TNF-α, and transforming growth factor (TGF)-β was observed in the hippocampus and cortex of adolescent PAE offspring (Tiwari and Chopra, 2011). Similarly, a paradigm of moderate gestational alcohol exposure via a liquid diet revealed significant PAE-related increases in TNF-α and Il-1β protein in the hippocampus of PAE offspring at PD30 (Wang et al., 2019). Furthermore, Chang et al. (2015) reported that gestational alcohol exposure increased basal chemokine CCL2 receptor (CCR2) mRNA expression in the lateral hypothalamus, as well as the density of CCR2+ neurons in this same region. Since experiments such as these have demonstrated the sensitivity of the adolescent brain to prenatal/perinatal alcohol exposure, one goal of the current experiments was to investigate whether PAE would alter steady-state levels of neuroimmune gene expression across several different brain regions later life, both during the critical period of adolescence, as well as in adulthood.

Beyond the basal neuroimmune state, other research has demonstrated that PAE affects the offspring's response to later insult, such as with an immune or alcohol challenge. For example, PAE has been shown to increase susceptibility to infectious disease throughout the lifespan (Gauthier, 2015), with PAE offspring also exhibiting increased vulnerability to chronic neuropathic pain, exacerbated inflammation to adjuvant-induced arthritis, and an altered immune response to an immunogen such as lipopolysaccharide (LPS; Bodnar et al., 2016; Noor and Milligan, 2018; Reid et al., 2019). Alcohol itself has also been shown to be a potent activator of a wide range of cytokines and other inflammation-related genes in adult animals (Crews et al., 2017; Deak et al., 2012; Erickson et al., 2019), with exposure to binge- or suprabinge-like doses of ethanol leading to widespread changes in the expression of neuroinflammatory markers in both the CNS and periphery (Crews et al., 2017). It is not surprising, then, that PAE would also affect the offspring's response to a later ethanol challenge. For instance, in a recent study, rats were exposed to alcohol from GD10–16 and then given an alcohol challenge in adulthood. While acute ethanol administration resulted in elevations of Il-6 in the cortex, these ethanol-induced increases were exacerbated in adult female PAE rats (Terasaki and Schwarz, 2017).

Importantly, evidence is accumulating to suggest that adolescence is associated with alterations in neuroimmune responsiveness to alcohol challenges that appear different from their adult counterparts. Evidence from our lab and others has shown that adolescent rats and mice (∼PD28–PD60) demonstrate reduced neuroimmune function following an acute challenge, regardless of whether the challenge consisted of ethanol, LPS, or stress (Deak et al., in preparation; Doremus-Fitzwater et al., 2015; Kane et al., 2014). In these studies, cytokine reactivity was severely impaired in adolescents, as well as the resultant activation of the hypothalamic-pituitary-adrenal (HPA) axis to the challenge (Girard-Joyal et al., 2015). Therefore, another goal of the current experiments was to examine potential differences in evoked responses of neuroimmune factors to a binge-like ethanol challenge in rats with or without PAE at two different developmental time points—adolescence and adulthood. These studies are especially important because adolescence is a developmental period that is characterized by increased alcohol consumption (Doremus et al., 2005; Vetter et al., 2007), with prenatal alcohol exposure exacerbating this adolescent-typical drinking behavior (Chang et al., 2015; Fabio et al., 2015; Pueta et al., 2008; Youngentob and Glendinning, 2009). PAE has been shown to increase ethanol acceptance by adolescent rats, an effect that seems to reflect a decreased aversion to, and an altered "tuning" of, neural responses to ethanol's component flavor qualities of bitter and oral irritation, as well as its odor (Glendinning et al., 2012, 2017; Middleton et al., 2009; Youngentob et al., 2007a, 2012). Thus, an examination of the interaction of PAE and adolescent ethanol challenge on neuroimmune responses could be of important functional significance for understanding the mechanisms by which PAE leads to future vulnerability to ethanol effects.

MATERIALS AND METHODS

Subjects

Timed pregnant Long-Evans dams were acquired from Envigo (formerly known as Harlan; Indianapolis, IN, USA) and shipped during the first week of gestation to SUNY Upstate Medical University (an AAALAC-accredited facility). Pregnant dams were housed under standard colony conditions (22°C; 12:00 h light:dark cycle, with lights on 06:00) with food and water available *ad libitum* at all times, except during the period of prenatal alcohol exposure described below. All experimental procedures were approved by the Committee on Humane Use of Animals (CHUA) at SUNY-Upstate Medical University (previous employer for SY), and studies were conducted following the Public Health Service (PHS) policy on the Humane Care and Use of Laboratory Animals.

Prenatal Alcohol Exposure

The procedures used for prenatal alcohol exposure were employed in previous studies (Middleton et al., 2009; Youngentob et al., 2007a) and are briefly described here. Dams were first assigned to one of two liquid diet conditions that were nutritionally balanced and equivalent to each other concerning their vitamin, mineral, protein, carbohydrate, fat, and fiber content: free-choice liquid (FCL) diet consumption or alcohol-containing liquid diet consumption. To begin, all pregnant dams were first weaned onto the liquid control diet (L10252 recipe; Research Diets, New Brunswick, NJ, USA) from gestational days (GD) 6–10. This diet provided 1.02 kcal/g. For dams in the FCL group, the diet was ethanol-free for the duration of the experiment. For dams producing pups in the PAE condition, however, the concentration of ethanol in the diet was gradually increased during this period (2.2% vol/vol on GD 6–8; 4.5% vol/vol on GD 9–10). From GD11, dams in the FCL group continued to have *ad libitum* access to the liquid diet and water until GD 20. In contrast, ethanol-drinking pregnant dams began receiving *ad libitum* access to a liquid diet containing 35% of daily calories from ethanol (6.7% ethanol vol/vol; total calories = 1.02 kcal/g) through GD 20 (L10251 recipe: Research Diets, New Brunswick, NJ, USA). Previous research using this

paradigm of gestational ethanol exposure has demonstrated that dams reach peak blood ethanol concentrations of approximately 150 mg/dl by GD 17 (Youngentob et al., 2007a).

Offspring

Litters were culled to a total of 10 pups per dam within 24 h of birth, with even numbers of males and females maintained where possible (i.e., no fewer than four, but no more than six, males or females per litter). At culling, pups were cross-fostered to dams fed the FCL diet. This is a standard procedure that ensured the effects of PAE (and other gestational manipulations) were not confounded by differences in maternal care displayed by PAE dams toward their pups. Litters were weaned on PD21, which included rehousing with a same-sex littermate until the time of the later drug challenge. To control for litter effects, no more than one male and one female offspring from each litter were placed into an experimental group.

Experimental Procedure

Two separate cohorts of offspring were used to examine neuroimmune responsiveness to acute ethanol intoxication at two different developmental periods: mid-adolescence (PD35) or adulthood (PD90). Thus, both cohorts consisted of a 2 (Diet: free-choice control liquid diet vs. ethanol liquid diet) × 2 (Drug Challenge: tap water vs. ethanol) × 2 (Sex: male vs. female) between-subjects factorial design (see **Figure 1**; for the adolescent cohort, $N = 79$ with $n = 9$–10 per group; for the adult cohort, $N = 75$ with $n = 8$–10). Following prenatal exposure to either the free-choice control or ethanol-containing diet, offspring were left to mature with their littermate until the postnatal ages selected for analysis. At the target ages (i.e., PD35 or PD90), rats were given an acute intragastric (i.g.) intubation of either tap water or ethanol (4 g/kg). Previous work from our laboratory has extensively studied the acute neuroinflammatory response to an acute ethanol challenge. After comparing multiple routes of exposure (i.p. vs. i.g.), multiple doses of ethanol (1–5 g/kg), and multiple time points (e.g., Gano et al., 2019; Doremus-Fitzwater et al., 2014, 2015), we have repeatedly demonstrated that a dose of 4 g/kg delivered i.g. will result in a consistent pattern of alterations in cytokine gene expression across a variety of brain regions. Importantly, these changes occur without the additional inflammatory activation that could potentially be caused by an i.p. injection. Moreover, these neuroinflammatory factors are changing in a way that is distinct from peripheral immune measures, thus indicating that these effects are specific to the brain. This 4 g/kg bolus consisted of ethanol (95%) mixed with tap water to a final concentration of 20% (v/v). Animals that received tap water alone were given their intubation at a volume equivalent to what rats in the ethanol condition received. Previous studies from our laboratory (Doremus-Fitzwater et al., 2014, 2018) have demonstrated that, with this dose and route of exposure, peak intoxication and acute neuroimmune alterations are reached approximately 3 h after ethanol administration. Hence, in both cohorts, samples were collected at this time point following the drug challenge.

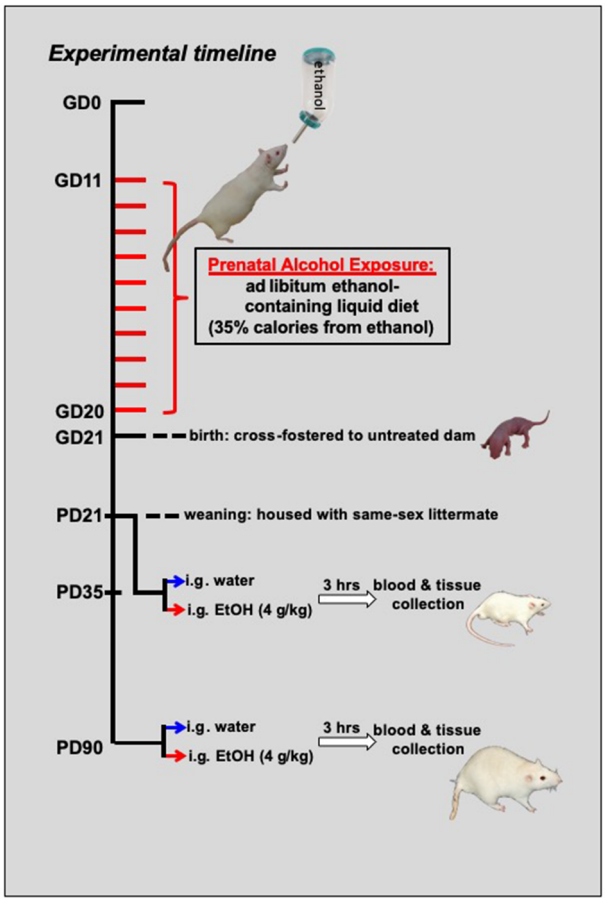

FIGURE 1 | Schematic representing the experimental timeline. From gestational day (GD) 11–GD20, pregnant dams were switched to a liquid diet. For half of the mothers, this diet was a control liquid that did not contain any alcohol, and it was provided *ad libitum*. For the other half of the dams, 35% of the calories in the liquid diet were from the addition of alcohol. At birth, offspring from these dams were cross-fostered to untreated dams and remained with their foster moms until the time of weaning at postnatal day (PD) 21. For the adolescent cohort ($N = 79$; $n = 9$–10 per group), these offspring matured until mid-adolescence, at which time they were acutely challenged with an intragastric (i.g.) gavage of either a binge-like dose of ethanol (4 g/kg) or tap water. Another cohort ($N = 75$; $n = 8$–10 per group) of rats matured into adulthood (P90) before being challenged with ethanol or tap water. For both age groups, trunk blood and brain tissue were collected at peak intoxication, 3 h after the administration of the drug challenge.

Tissue Collection and Processing

Three hours after drug challenge, rats were euthanized by brief CO_2 exposure. Following decapitation, trunk blood was collected in K3-EDTA containing glass blood collection tubes (BD Vacutainers, VWR cat. no. VT6450, Radnor, PA, USA), with plasma then separated in a refrigerated centrifuge and frozen at −20°C until time of assay. Whole brains were immediately flash frozen on dry ice and stored at −80°C. Brain structures of interest [e.g., hippocampus, amygdala, and paraventricular nucleus of the hypothalamus (PVN)] were collected by microdissection. These regions were chosen for analysis because of numerous prior studies reporting sensitivity of these areas to PAE neuroimmune

effects (Bodnar et al., 2016; Cantacorps et al., 2017; Drew et al., 2015; Terasaki and Schwarz, 2016), as well as their responsiveness to acute ethanol challenge on neuroimmune functioning (Doremus-Fitzwater et al., 2014, 2015, 2018; Gano et al., 2017; Kane et al., 2014; Terasaki and Schwarz, 2017). To do this, frozen brains were sliced in a cryostat (maintained at −20°C), with brain regions collected using chilled micropunches (1.0–2.0 mm) according to the Paxinos and Watson (1998) rat brain atlas (see **Figure 2**). Brain punches were collected from the right side of each structure and were stored at −80°C until the time of RNA extraction.

Reverse-Transcription Polymerase Chain Reaction

A Qiagen TissueLyser (Qiagen, Valencia, CA, USA) provided rapid, thorough, and consistent homogenization of brain samples. Each structure was placed into a 2.0 ml Eppendorf tube containing 500 μl of Trizol® RNA reagent (Invitrogen, Grand Island, NY, USA) and a 5 mm stainless steel bead, and was then rapidly shaken for 2 min for complete disruption/homogenization of the tissue. Chloroform (100 μl) was then added to the Trizol solution, the samples briefly were shaken, and then samples were centrifuged for 15 min at 4°C. An equal volume of 70% ethanol was added to the supernatant and purified through RNeasy mini columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Columns were washed with buffer and eluted with 30 μl of RNase-free water (65°C). RNA yield and quality were determined using a Nanodrop micro-volume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA), with total RNA stored at −80°C until the time of cDNA synthesis. Synthesis of cDNA was performed on 0.1–1.0 μg of normalized total RNA from each sample using the QuantiTect® Reverse Transcription Kit (Cat. No. 205313, Qiagen, Valencia, CA, USA) which included a DNase treatment step. All cDNA was stored at −20°C until the time of assay.

Probed cDNA amplification was performed in a 10 μl reaction consisting of 5 μl IQ SYBR Green Supermix (Bio-Rad, cat. no. 170-8882, Hercules, CA, USA), 0.5 μl primer (final concentration 250 nM), 0.5 μl cDNA template, and 4 μl Rnase-free water run in triplicate in a 384 well plate (BioRad, cat. no. HSP-3805), and captured in real-time using a PCR detection system (BioRad, model no. CFX384). Following a 3-min hot start (95°C), samples underwent denaturation for 30 s at 95°C, annealing for 30 s at 60°C and extension for 30 s at 72°C for 50 cycles. An additional denaturation (95°C, 1 min) and annealing cycle (55°C, 1 min) were conducted to ensure proper product alignment before melt curve analysis. For melt curve analysis, samples underwent 0.5°C changes every 15 s ranging from 55°C to 95°C. A single peak expressed as the negative first derivative of the change in fluorescence as a function of temperature indicated primer specificity to the target gene. Glyceraldehyde 3-phosphate dehydrogenase (*Gadph*) was used as a reference gene in these experiments, as studies from our laboratory have revealed more stable gene expression across ethanol treatment conditions with this gene (Doremus-Fitzwater et al., 2014, 2015; Gano et al., 2017). Before conducting analyses of cytokine data, *Gapdh* expression was first examined as a separate target to confirm no statistical differences in expression of this reference gene across conditions. Thereafter, gene expression of neuroinflammatory targets was quantified relative to the expression of *Gapdh* using the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen, 2001), with male, FCL, vehicle-challenged controls serving as the ultimate control group. Specifically, the equation used was: $2^{-\Delta\Delta C(t)}$ target gene–C(t) *Gapdh* for individual) − (mean of ultimate control group: C(t) target gene–C(t) *Gapdh*)] × 100. Thus, in all figures and tables showing inflammatory genes, this equation was used to calculate relative gene expression. A list of genes examined and their primer sequences can be found in **Table 1**.

Plasma Measurement of Blood Ethanol Concentrations and Corticosterone

All blood ethanol concentrations (BECs) were determined in 5 μl aliquots of plasma using an Analox AM-1 alcohol analyzer (Analox Instruments, Lunenburg, MA, USA). The machine was first calibrated using a 100 mg% industry-standard, with

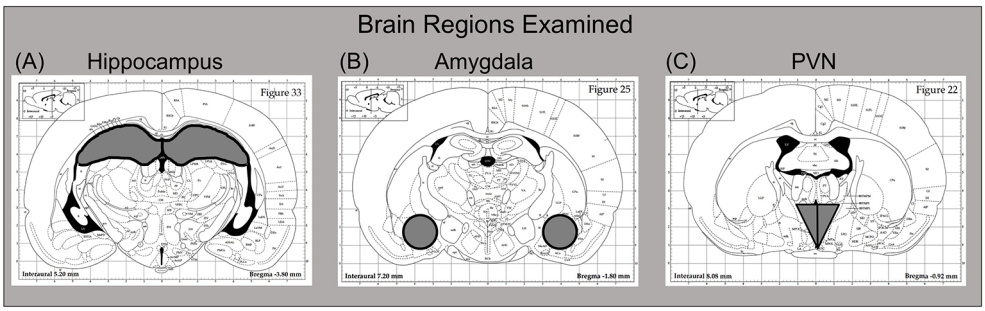

FIGURE 2 | Dissection guide for sites of interest, with images modified from the Paxinos and Watson (1998) rat brain atlas (Published with permission by Elsevier). The **(A)** hippocampus **(B)** amygdala, and **(C)** paraventricular nucleus of the hypothalamus (PVN) were collected for analysis. For the hippocampus and amygdala, the right side of the structure was used for these reverse transcription-polymerase chain reaction (RT-PCR) assays, with the left side harvested for other purposes. In the case of the PVN, the entire punch that was collected was used here for RT-PCR.

TABLE 1 | Reverse transcription-polymerase chain reaction (RT-PCR) primers and sequences for genes examined in brain.

Gene target	Accession #	RT-PCR Primer sequences
Il-6[a]	NM_012589.2	Forward: 5′-TAGTCCTTCCTACCCCAACTTCC-3′ Reverse: 5′-TTGGTCCTTAGCCACTCCTTC-3′
IκBα[b]	NM_001105720.2	Forward: 5′-CTGTTGAAGTGTGGGGCTGA-3′ Reverse: 5′-AGGGCAACTCATCTTCCGTG-3′
Il-1β[c]	NM_031512.2	Forward: 5′-TCCTCTGTGACTCGTGGGAT-3′ Reverse: 5′-TGGAGAATACCACTTGTTGGCT-3′
Tnfα;[d]	NM_012675.3	Forward: 5′-GTCCCAACAAGGAGGAGAAGTT-3′ Reverse: 5′-CTCCGCTTGGTGGTTTGCTA-3′
CX_3CL-1[e]	NM_134455.1	Forward: 5′-GCCATCATCCTGGAGACGAG-3′ Reverse: 5′-CGCTTCTCAAACTTGCCACC-3′
CX_3CL-1R[f]	NM_133534.1	Forward: 5′-TCTTCCTCTTCTGGACGCCT-3′ Reverse: 5′-TAAACGCCACTGTCTCCGTC3′
Gapdh[g]	NM_017008	Forward: 5′-GTGCCAGCCTCGTCTCATAG-3′ Reverse: 5′-AGAGAAGGCAGCCCTGGTAA-3′

[a]*Interleukin-6.* [b]*Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.* [c]*Interleukin-1 beta.* [d]*Tumor necrosis factor-alpha.* [e]*Chemokine (C-X3-C motif) ligand 1 (also known as fractalkine).* [f]*Chemokine (C-X3-C motif) ligand 1 receptor (also known as fractalkine receptor).* [g]*Glyceraldehyde 3-phosphate dehydrogenase.*

BECs recorded in milligrams per deciliter (mg%). Accuracy was confirmed with a quality control solution provided by Analox Instruments, which contained a known concentration of ethanol. After confirmation with the quality control, experimental samples were measured and counterbalanced across groups concerning the order in which they were processed. Accuracy of the machine was systematically rechecked by reading the quality control following the measurement of every 12–15 samples, as well as after the final sample.

Quantitative determination of plasma CORT was assessed by a commercially available ELISA kit (Cat No: ADI-901-097; Enzo Life Sciences, Farmingdale, NY, USA). The CORT assay had a sensitivity of 27.0 pg/ml and an inter-assay coefficient of 10.29%. The samples were diluted 1:30 and heat-inactivated to denature endogenous corticosteroid-binding globulin (CBG) by immersion in 75°C water for 60 min, which produces a much more reliable and uniform denaturation of CBG than the enzyme cleavage step provided by the kit (unpublished observations). After heat inactivation of CBG, samples were processed according to the directions provided by the kit.

Data Analysis

Because rats challenged at PD35 vs. PD90 were obtained from two separate cohorts, data from these two age groups were analyzed separately. Thus, while general patterns of ethanol and PAE effects can be compared across ages, these data sets did not allow for direct statistical comparison between adolescents and adults.

For all variables of interest, data were first checked for outliers using the extreme studentized deviate (ESD) method (Grubb's test), with values outside the boundaries of more or less than two standard deviations from the group mean meeting the criterion for outliers. Given the logarithmic amplification of RT-PCR data, it is not usual that there are instances in which a sample was an extreme data point in the analysis of only one target within a structure. In these situations, the data point was only removed in the analysis of that particular dependent variable. However, if a sample was an outlier for more than two individual gene targets, it was then dropped for analysis of all targets in that structure. Analyses of the adolescent data included four outliers or missing data points: one BEC sample from a male PAE ethanol-challenged rat was lost during processing; one female FCL ethanol-challenged rat was an outlier for all targets in the hippocampus; one male FCL ethanol-challenged animal was an outlier for all gene targets in the amygdala, and one female PAE water-challenged rat was not included in the analyses of all targets in the PVN. For analyses of adult data, gene targets in the PVN only revealed one outlier—a male PAE vehicle-challenged rat was eliminated from the analysis of *IκBα* in this tissue compartment. For the adult amygdala data, three rats were eliminated from analyses of all amygdala gene targets: one female PAE rat challenged with water, one male PAE rat challenged with ethanol, and one female FCL animal challenged with ethanol. Additionally, in the analysis of *IκBα* in the amygdala, a female FCL vehicle-exposed rat was excluded. During processing the of hippocampal tissue one sample from the female FCL ethanol-challenged group was lost. Analyses of outliers for gene targets in the hippocampus involved the exclusion of several outliers for all genes: one male and one female from the FCL vehicle-challenged groups, as well as one male from the PAE water-exposed condition. For *Il-1β, Tnfα,* and *Il-6* analyses, an additional male FCL water-exposed rat was also excluded, whereas an additional male PAE water-exposed and female FCL water-exposed rat were also excluded for *Tnfα,* and *Il-6* analyses, respectively. In the analyses of cytokine targets, plasma corticosterone concentrations, and plasma ethanol concentrations, data were analyzed (Statistica, TIBCO®) using a 2 (Prenatal Diet: FCL vs. PAE) × 2 (Sex: Male vs. Female) × 2 (Drug Challenge: Veh vs. EtOH) factorial ANOVA ($p < 0.05$), with Fisher's Least Significant Difference (LSD) test used for *post hoc* examination of any significant 2- or 3-way interactions ($p < 0.05$) that were observed.

RESULTS

For both adolescent and adult rats, results from the analyses of all gene targets that were examined across three different

TABLE 2 | Group means and SEMs for neuroimmune targets in adolescent and adult rats following a water or ethanol challenge.

	Adolescents				Adults			
	FCL		PAE		FCL		PAE	
	VEH	EtOH	VEH	EtOH	VEH	EtOH	VEH	EtOH
Hippocampus								
Il-1β[a]	96.7 (8.6)	**68.8 (6.7)**	94.3 (13.8)	**60.7 (8.0)**	109.3 (8.2)	**69.9 (8.7)**	102.9 (9.3)	**53.3 (5.6)**
Tnfα[b]	101.4 (8.9)	**61.3 (11.1)**	92.2 (8.8)	**47.8 (6.2)**	M: 103.9 (11.6)	**M: 34.9 (5.9)**	*M: 112.0 (18.0)*	**M: 71.9 (11.6)**
					F: 166.7 (17.1)	**F: 59.0 (8.7)**	*F: 101.4 (13.0)*	**F: 58.5 (8.70)**
CX_3CL-1[c]	105.2 (4.4)	105.1 (3.3)	104.4 (5.0)	102.0 (3.2)	M: 91.1 (7.6)	**M: 79.9 (8.6)**	M: 99.6 (6.1)	**M: 86.0 (5.3)**
					F: 104.0 (6.8)	**F: 96.6 (8.2)**	F: 93.3 (5.7)	**F: 75.9 (8.0)**
CX_3CL-1R[d]	102.4 (3.4)	**95.4 (2.8)**	*99.9 (4.6)*	**85.1 (2.3)**	M: 104.7 (9.9)	**M: 81.5 (10.5)**	M: 115.7 (6.5)	**M: 88.5 (5.9)**
					F: 120.3 (8.7)	**F: 95.2 (8.2)**	F: 102.2 (5.9)	**F: 81.3 (10.3)**
Amygdala								
Il-1β	112.3 (12.5)	**75.1 (5.8)**	108.7 (11.5)	**64.2 (5.0)**	116.6 (10.0)	**87.5 (10.4)**	111.0 (10.3)	**76.2 (7.5)**
Tnfα	109.7 (8.9)	**65.4 (4.5)**	107.9 (8.6)	**51.7 (5.4)**	101.1 (7.2)	**35.0 (3.8)**	106.8 (7.9)	**49.1 (4.3)**
CX_3CL-1	104.5 (3.4)	96.9 (3.8)	99.7 (4.3)	104.7 (6.1)	M: 102.0 (7.2)	M: 112.5 (4.4)	M: 102.6 (8.6)	M: 98.8 (7.3)
					F: 145.3 (19.6)	F: 122.3 (10.1)	F: 143.5 (12.8)	F: 106.0 (9.4)
CX_3CL-1R	106.8 (3.5)	100.9 (3.4)	116.4 (4.5)	105.0 (6.3)	109.0 (5.8)	**91.2 (4.4)**	116.8 (7.1)	**98.9 (5.3)**
PVN								
Il-1β	*M* : 102.0 (6.7)	**M: 64.1 (6.0)**	M: 101.0 (10.6)	**M: 79.1 (6.5)**	107.8 (10.5)	**88.7 (9.4)**	*95.9 (7.3)*	**66.0 (5.8)**
	F : 100.0 (7.1)	**F: 75.2 (7.2)**	F: 79.1 (7.2)	**F: 65.6 (7.2)**	107.8 (10.5)	**88.7 (9.4)**	*95.9 (7.3)*	**66.0 (5.8)**
Tnfα	*M* : 102.1 (6.5)	**M: 62.0 (4.4)**	M: 92.8 (8.1)	**M: 60.3 (5.4)**	94.4 (8.7)	**43.5 (4.1)**	89.4 (5.2)	**51.6 (4.4)**
	F : 107.8 (8.4)	**F: 50.5 (5.1)**	F: 80.4 (8.0)	**F: 61.9 (8.1)**				

The effects of prenatal alcohol exposure (PAE) on basal and ethanol-evoked neuroimmune gene expression were assessed in either adolescence or adulthood. Control (free-choice liquid diet; FCL) and PAE-exposed pups matured until adolescence (P35) or adulthood (P90), at which time they were challenged with an acute gavage of either water (VEH) or ethanol (EtOH) (4 g/kg). Three hours after drug challenge, brains were collected for gene expression assessment in the hippocampus, amygdala, and paraventricular nucleus of the hypothalamus (PVN). Values shown are means for each group, with standard error of the mean (SEM) shown in parentheses. Gene abbreviations: [a]interleukin-1 beta; [b]tumor necrosis factor alpha; [c]chemokine (C-X3-C motif) ligand 1 (also known as fractalkine); [d]chemokine (C-X3-C motif) ligand 1 receptor (also known as fractalkine receptor). In these analyses, there were no instances in which a 3-way interaction between Sex, PAE, and Ethanol Challenge were observed. Since the majority of significant effects involved main effects of Ethanol Challenge or PAE, this table highlights these differences by indicating main effects of EtOH challenge with bold-faced text, and significant main effects of PAE with italics. However, there were a few analyses in which a significant 2-way interaction involving Sex and either PAE or Ethanol Challenge were observed. In order to show these Sex differences, means for males and females are presented separately in the table. In doing so, the inclusion of eight group means make highlighting differences from post hoc analysis of these 2-way interactions confusing to interpret in table format. Thus, additional formatting is not included for interactions involving Sex beyond group means.

brain regions are in **Table 2**. A description of these significant outcomes follows below.

Ethanol Challenge-Induced Alterations in Cytokine Expression

Acute challenge with a binge-like dose of ethanol resulted in significant increases in expression of *Il-6* (see **Figure 3**) in all three brain regions examined and at both ages (main effect of Drug Challenge at PD35 for *Il-6* in hippocampus: $F_{(1,70)} = 42.21$, $p \leq 0.000001$, amygdala: $F_{(1,70)} = 26.71$, $p \leq 0.00001$, and PVN: $F_{(1,70)} = 35.76$, $p \leq 0.000001$; main effect of Drug Challenge at PD90 for *Il-6* in hippocampus: $F_{(1,61)} = 23.95$, $p \leq 0.00001$; amygdala: $F_{(1,62)} = 43.98$, $p \leq 0.00001$; and PVN: $F_{(1,65)} = 49.33$, $p \leq 0.000001$). Similarly, an ethanol-related increase in expression of *IκBα* (see **Figure 4**) was also observed at PD35 and at PD90 in all brain areas analyzed [main effect of Drug Challenge at PD35 for *IκBα* in hippocampus: $F_{(1,70)} = 11.49$, $p \leq 0.001$, amygdala: $F_{(1,70)} = 13.33$, $p \leq 0.001$, and PVN: $F_{(1,70)} = 20.11$, $p \leq 0.0001$; main effect of Drug Challenge at PD90 for *IκBα* in hippocampus: $F_{(1,61)} = 6.62$, $p \leq 0.05$; amygdala: $F_{(1,54)} = 18.35$, $p \leq 0.00001$; and PVN: $F_{(1,64)} = 31.89$, $p \leq 0.000001$]. Although the stimulatory effect of ethanol challenge on hippocampal *IκBα* expression seemed less robust among the female offspring (especially the PAE females), Sex did not significantly interact with this ethanol effect. Furthermore, in none of these instances did the effects of ethanol challenge interact with Sex or Prenatal exposure to ethanol.

In contrast to the ethanol-related increases in gene expression noted above, in some cases, acute ethanol challenge resulted in significant reductions in neuroinflammatory gene expression. For example, expression levels of *Il-1β* (**Table 2**) were significantly suppressed among both adolescents and adults during acute intoxication in the hippocampus (main effect of Drug Challenge for adolescents: $F_{(1,70)} = 9.99$, $p \leq 0.01$; for adults: $F_{(1,61)} = 32.80$, $p \leq 0.00001$), amygdala (for adolescents: $F_{(1,70)} = 19.98$, $p \leq 0.0001$; for adults: $F_{(1,62)} = 10.83$, $p \leq 0.001$), and PVN (for adolescents: $F_{(1,70)} = 22.51$, $p \leq 0.0001$; for adults: $F_{(1,65)} = 8.18$, $p \leq 0.01$). The analysis of *Tnfα* expression levels also revealed an ethanol-associated decrease in all 3 brain areas examined (**Table 2**), with both adolescents (main effect of Drug Challenge in the hippocampus $F_{(1,70)} = 25.12$, $p \leq 0.00001$; amygdala $F_{(1,70)} = 49.78$, $p \leq 0.00001$; and PVN $F_{(1,70)} = 58.95$, $p \leq 0.000001$) and adults (main effect of Drug Challenge in the hippocampus $F_{(1,61)} = 59.61$, $p \leq 0.000001$; amygdala $F_{(1,62)} = 97.04$, $p \leq 0.00001$; and PVN $F_{(1,65)} = 55.93$, $p \leq 0.000001$) exhibiting this suppression following ethanol challenge. When expression levels of *Fractalkine* (*CX_3CL-1*) and *Fractalkine receptor* (*CX_3CL-1R*) were examined in the hippocampus and amygdala (**Table 2**), again, acute ethanol intoxication generally showed patterns of decreased gene expression that were small in magnitude

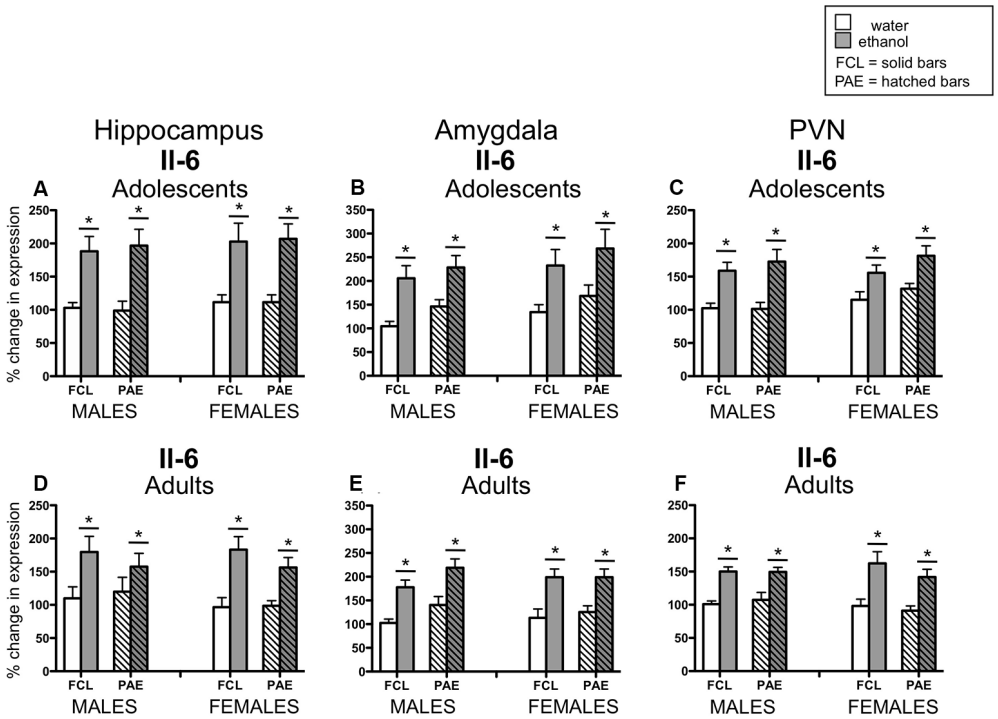

FIGURE 3 | Adolescent (top row) and adult (bottom row) male and female rats were given an acute intragastric (i.g.) challenge of tap water (white bars) or 4-g/kg ethanol (EtOH; 20% v/v; gray bars), with brains collected for analysis 3 h after intubation. Half of the animals were offspring from mothers exposed to a prenatal alcohol diet (PAE groups; hatched bars), whereas the other half of the animals were born to dams that experienced a free-choice liquid diet (FCL groups; solid bars). *Interleukin (Il)-6* gene expression was examined in the hippocampus (panels **A,D**), amygdala (panels **B,E**) and paraventricular nucleus of the hypothalamus (PVN; panels **C,F**), with data calculated as a relative change in gene expression using the $2^{-\Delta\Delta C(t)}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene and adult, male, FCL, water-exposed rats were used as the ultimate control group. Bars denote group means ± standard error of the mean (represented by vertical error bars). Data for adolescents and adults were analyzed separately, with the main effects of Drug Challenge signified by a horizontal line with an asterisk (*) above.

but statistically significant. For adolescents, acute ethanol slightly but significantly suppressed *CX_3CL-1R* expression, but only in the hippocampus (main effect of Drug Challenge: $F_{(1,70)} = 9.94, p \leq 0.01$). Adults likewise demonstrated an ethanol-induced reduction in *CX_3CL-1R* expression in hippocampus ($F_{(1,63)} = 15.87, p \leq 0.001$) and amygdala ($F_{(1,62)} = 9.12, p \leq 0.01$). Additionally, adults exhibited significant reductions in *CX_3CL-1* levels in the hippocampus ($F_{(1,63)} = 5.92, p \leq 0.05$), with *post hoc* analysis revealing that only adult females demonstrated a significant reduction in this gene in the amygdala [Sex × Drug Challenge: $F_{(1,62)} = 4.32, p \leq 0.05$].

Effects of Prenatal Alcohol Exposure on Neuroimmune Gene Expression

Although this model of prenatal ethanol exposure did not significantly interact with the stimulatory effects of ethanol challenge on *Il-6* and *IκBα* expression, ethanol-induced suppression of *Tnfα* was impacted by PAE in the PVN for adolescents (PAE × Drug Challenge interaction: $F_{(1,70)} = 5.76, p \leq 0.05$), as well as in the hippocampus for adults (PAE × Drug Challenge interaction: $F_{(1,61)} = 7.77, p \leq 0.01$; see **Table 2**). *Post hoc* analysis demonstrated that, among adolescents, PAE reduced *Tnfα* levels in water-challenged controls, which led to a less marked reduction in expression of *Tnfα* when this group was compared to the PAE ethanol-challenged rats. A comparable pattern of changes was observed for *Tnfα* expression in the hippocampus for adults. Thus, in the limited cases in which PAE affected ethanol alterations in neuroinflammatory markers, it blunted ethanol-related suppression of gene expression.

Overall, PAE effects were more often statistically observed in adults compared to adolescents, and the magnitude of the PAE effects was less marked than those induced by ethanol challenge. A main effect of PAE was revealed for adults when *Il-1β* expression in the PVN was analyzed (**Table 2**; $F_{(1,65)} = 4.19, p \leq 0.05$), and also for adolescents when *CX_3CL-1R* expression in the hippocampus was examined (**Table 2**; $F_{(1,70)} = 9.94, p \leq 0.05$). Moreover, these PAE effects often interacted with Sex. For example, in adults, there was a significant Sex × PAE interaction in the hippocampus for *Tnfα* ($F_{(1,61)} = 10.86, p \leq 0.01$), *$CX_3CL$-1* ($F_{(1,63)} = 5.06, p \leq 0.05$) and *$CX_3CL$-1R* ($F_{(1,63)} = 4.26, p \leq 0.05$) expression (**Table 2**). For all three of these gene targets, the Sex x PAE interaction demonstrated that, among males, prenatal alcohol exposure augmented expression when compared to the FCL controls. In contrast, PAE suppressed expression of these genes for female PAE offspring compared to

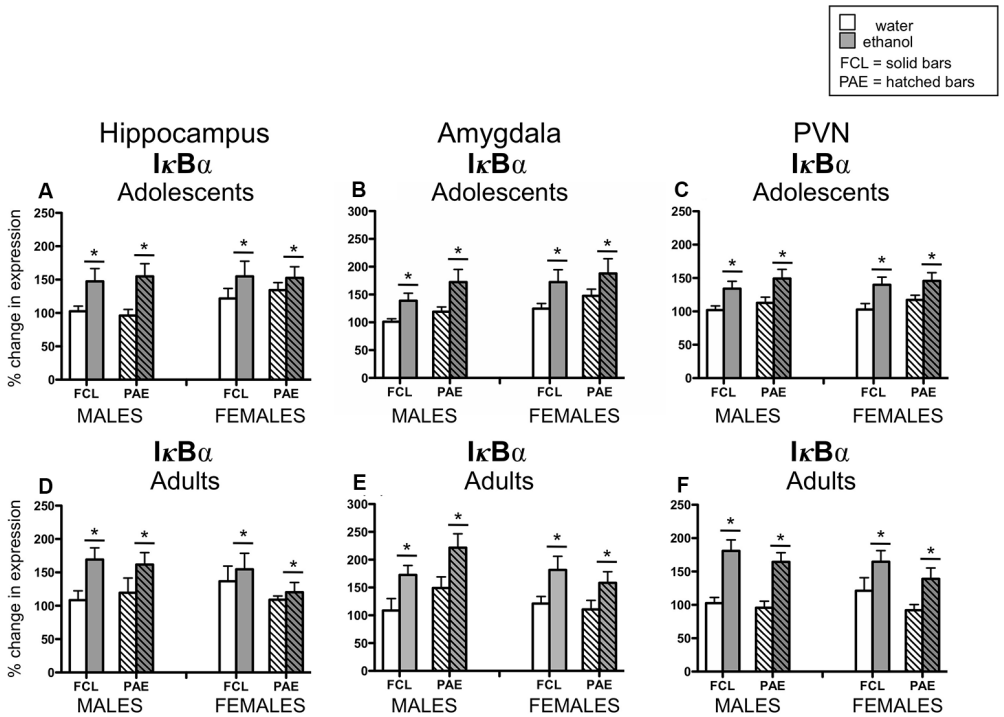

FIGURE 4 | Adolescent (top row) and adult (bottom row) male and female rats were given an acute intragastric (i.g.) challenge of tap water (white bars) or 4-g/kg ethanol (EtOH; 20% v/v; gray bars), with brains collected for analysis 3 h after intubation. Hatched bars represent offspring born to mothers exposed to a prenatal alcohol diet (PAE groups), whereas solid bars denote offspring from dams that experienced a free-choice liquid diet (FCL groups). Expression of *IκBα* was examined in the hippocampus (panels **A,D**), amygdala (panels **B,E**) and paraventricular nucleus of the hypothalamus (PVN; panels **C,F**), with data calculated as a relative change in gene expression using the $2^{-\Delta\Delta C(t)}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene and adult male FCL offspring challenge with water used as the ultimate control group. Bars denote group means ± standard error of the mean (represented by vertical error bars). Data for adolescents and adults were analyzed separately, with the main effects of Drug Challenge indicated by a horizontal line with an asterisk (*) above.

FCL offspring. The same significant pattern was observed in the Sex x PAE interaction for *IκBα* expression in the amygdala in adults ($F_{(1,54)} = 4.68$, $p \leq 0.05$) and for *Il-1*β expression in the PVN for adolescents ($F_{(1,70)} = 4.57$, $p \leq 0.05$).

Plasma Blood Ethanol and Corticosterone Concentrations

In the analyses of BECs, only ethanol-exposed rats were included. However, plasma samples collected from vehicle-challenged rats were still measured in the ANALOX and these readings confirmed no measurable amount of ethanol in their blood (i.e., all vehicle controls exhibited values at the floor of the assay's sensitivity; data not shown). Whereas adolescent rats challenged with ethanol had similar BECs regardless of their sex or prenatal condition (**Figure 5A**), adult female rats exhibited slightly but significantly higher BECs than their male counterparts after ethanol exposure (**Figure 5B**; main effect of Sex for adults: $F_{(1,35)} = 4.15$, $p \leq 0.05$).

When plasma corticosterone concentrations were assessed (**Figures 5C,D**), a significant sex difference was observed for both adolescents and adults, with females exhibiting generally higher levels of corticosterone than males (main effect of Sex for adolescents: $F_{(1,71)} = 4.34$, $p \leq 0.05$; for adults: $F_{(1,67)} = 7.77$, $p \leq 0.000001$). Furthermore, adolescents challenged with ethanol had a significantly greater corticosterone response at this time point compared to rats that received the vehicle intubation (main effect of Drug Challenge for adolescents: $F_{(1,71)} = 4.30$, $p \leq 0.05$).

DISCUSSION

Prenatal alcohol exposure leads to deficits in behavior and alterations in brain structure and function that are comparable in both humans and preclinical models. Additionally, PAE has been shown to alter neuroimmune function in offspring across development, with immediate and lasting changes in immune processes potentially contributing to the neurobehavioral consequences of FASD. To explore possible effects of PAE on both basal and ethanol-evoked cytokine responses in the CNS, the current experiments examined expression levels of several neuroimmune factors in the hippocampus, amygdala, and PVN of male and female PAE offspring at two developmental periods: mid-adolescence and adulthood. While ethanol was a potent modulator of neuroimmune factors in all three brain regions, the effects of PAE were much less marked in comparison. Furthermore, in most cases, PAE did not significantly influence ethanol-induced changes

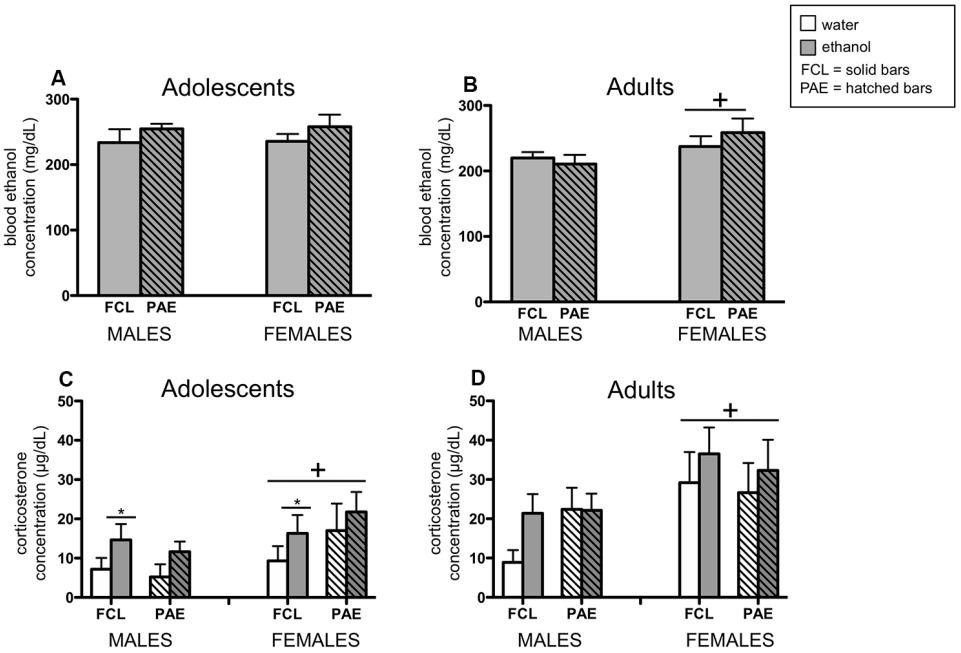

FIGURE 5 | Plasma concentrations of ethanol (panels **A,B**) and corticosterone (panels **C,D**) were examined 3 h after an acute ethanol (4 g/kg; gray bars) or tap water (white bars) gastric gavage. Adolescents (left panels) and adults (right panels) were male and female offspring from mothers that experienced either a liquid diet containing ethanol (PAE groups; hatched bars) or a control liquid diet (FCL groups; solid bars) during gestational days 11–20. Bars denote group means ± standard error of the mean (represented by vertical error bars). Data for adolescents and adults were analyzed separately, and only ethanol-challenged rats included in the analyses of blood ethanol concentration. A plus symbol (+) indicates a significant main effect of Sex within a particular age group, whereas the line with an asterisk (*) denotes a significant main effect of the Drug Challenge.

in any of the age or sex groups examined when they were compared to offspring that did not have gestational exposure to alcohol.

In the present experiments, adolescent and adult rats were challenged with a binge-like dose of ethanol, and then the expression of several neuroimmune factors assessed during peak intoxication. Ethanol markedly and significantly changed expression levels of the cytokines examined, with the results of this acute ethanol challenge confirming what we have previously observed (Doremus-Fitzwater et al., 2015, 2018; Gano et al., 2016, 2017). More specifically, ethanol intoxication elevated expression levels of *Il-6* and IκBα in all three brain regions examined, yet attenuated the expression of *Tnf*α and *Il-1*β mRNA. We have termed this ethanol intoxication-induced pattern of cytokine changes "rapid alterations in neuroimmune gene expression" (RANGE; Gano et al., 2016, 2017), and we have observed such changes in multiple strains of rats (Gano et al., 2017), at multiple ages (Doremus-Fitzwater et al., 2015; Gano et al., 2017), and in both sexes (Gano et al., 2017). Whereas repeated binge administrations of ethanol have been reported to lead to a state of heightened neuroimmune activation that is responsible for neuroinflammatory brain damage and some of the behavioral and cognitive effects of ethanol (Crews et al., 2017; Alfonso-Loeches et al., 2011; Montesinos et al., 2016), our laboratory and others have shown that a different pattern of responses is observed during the first few intoxicating exposures to ethanol challenge (Doremus-Fitzwater et al., 2015, 2018; Gano et al., 2016, 2017; Terasaki and Schwarz, 2017). While we have not yet identified the mechanisms responsible for these acute intoxication-related alterations in cytokines, acute ethanol exposure likely represents a non-pathogenic challenge that induces a sterile inflammatory response via activation of TLR4s through danger-associated molecular patterns (DAMPs), such as heat-shock proteins (e.g., hsp72) or high-mobility group box 1 (HMGB-1; Whitman et al., 2013; Crews et al., 2017).

Regardless of the mechanisms leading to ethanol intoxication-associated changes in brain cytokines, PAE effects were less pronounced overall. Only a few significant instances of PAE effects were observed: in adults, PAE led to slight but significant reductions in *Il-1*β expression in the PVN in adults, and *CX$_3$CL-1R* expression in the hippocampus of PAE adolescents. Furthermore, PAE did not substantially impact ethanol-evoked responses, with the limited cases in which PAE significantly affected ethanol alterations in neuroinflammatory markers demonstrating that PAE blunted ethanol-related suppression of gene expression. Although not significant, it is worth noting that there was also a subtle but consistent augmentation of ethanol-induced increases in *Il-6* and *IκBα* expression in male and female adolescents and male adults. Using the same exposure model as in the present study, we have previously demonstrated alterations in ethanol-induced chemosensory plasticity that are important fundamental contributors to postnatal avidity for ethanol. For

example, using this model, young rats exposed to gestational ethanol show enhanced ethanol intake (Youngentob et al., 2007b), as well as behavioral responses to ethanol odor that were mediated, in part, by an effect of maternal ethanol treatment on the neural response of the olfactory epithelium (Youngentob et al., 2007a). Moreover, prior fetal exposure increased EtOH intake, in part, by decreasing the generally aversive flavor properties of ethanol's quinine-like bitter taste, capsaicin-like oral burning sensation, and aversive odor attributes (Glendinning et al., 2012; Youngentob and Glendinning, 2009). More recently, we have shown that fetal alcohol-induced attenuation in orosensory behavioral responses to later ethanol exposure is mediated by a reduction in the responsiveness of taste nerves and trigeminal chemosensory neurons to ethanol and its flavor components (Glendinning et al., 2017). Given the multitude of PAE effects observed with this model, and prior studies using heavier, binge-like, models of prenatal alcohol, we predicted pronounced PAE effects in the present study, which ultimately were not observed. However, more recently, researchers have suggested that low-to-moderate levels of PAE may result in subtle alterations in steady-state neuroinflammation that may not be observed under basal conditions. Indeed, the present experiments reported relatively few changes in basal neuroimmune gene expression that persisted into adolescence and adulthood with this relatively moderate PAE exposure paradigm, similar to a recent report (Terasaki and Schwarz, 2017)). Instead, it has been suggested that a subsequent perturbation, such as an immune or ethanol challenge (Bodnar et al., 2016; Noor and Milligan, 2018; Noor et al., 2017; Sanchez et al., 2017; Terasaki and Schwarz, 2016, 2017), may be required to unmask effects of prior PAE. In particular, an ethanol challenge during a later critical ontogenetic period such as adolescence has been thought to result in a situation in which these subtle lingering PAE effects might become visible, thus leading to increased vulnerability to ethanol-induced effects that might contribute to increased ethanol acceptance and drinking behaviors. However, in the present experiments, the PAE offsprings' neuroimmune response to the drug challenge was not as different from control offspring as expected. One possibility for this outcome is that the single acute ethanol challenge administered here was not optimal for revealing such prenatal-postnatal interactions based on the dose utilized (i.e., we may be observing a ceiling effect after a 4 g/kg administration) or time point examined (i.e., PAE may alter the kinetics of the immune response to acute challenge and we did not select an optimal time to capture PAE *vs*. FCL effects in response to the challenge). To fully reveal lingering PAE effects, repeated exposures to ethanol might be required to observe more significant consequences of PAE (e.g., similar to the two consecutive ethanol binges in Terasaki and Schwarz, 2017), or there may be other time points (e.g., withdrawal; Topper et al., 2015) at which PAE effects would be more evident. Furthermore, it is possible that other brain regions, such as the prefrontal cortex (Terasaki and Schwarz, 2017), might be more sensitive to lasting effects of PAE on cytokines and chemokines, and their responsiveness to acute ethanol.

When the effects of PAE were compared in males and females, there were several instances in which the direction of the influence of PAE on gene expression levels was opposite in males *vs*. females. For adults, the hippocampus was especially differentially affected by PAE across sex—*Tnfα, CX$_3$CL-1* and *CX$_3$CL-1R* expression was potentiated in PAE males compared to FCL males, whereas PAE females exhibited decreased expression of these genes relative to control offspring. Expression levels of *IκBα* in the amygdala in adults, and *Il-1β* levels in the PVN in adolescents revealed this same pattern. That females and males showed different responses to PAE-induced changes in neuroimmune factors was not unexpected. Under normal conditions, developmental differences in neuroimmune system function are present, including sex differences in microglial colonization and structure, and expression of neuroinflammatory factors (Schwarz and Bilbo, 2012; Schwarz et al., 2012). Additionally, other laboratories have reported male *vs*. female differences in the effects of PAE on both basal and challenge-evoked alterations in cytokines and chemokines (Terasaki and Schwarz, 2016, 2017; Topper et al., 2015). These reports have been somewhat equivocal, however, with females sometimes exhibiting augmented responses and in other instances females demonstrating blunted responses. Likely, differences in PAE duration and dosing, as well as the timing of assessment, brain region of interest, and type of developmental challenge are all variables contributing to contrasting sex differences across studies.

When considering the effects of ethanol on the neuroimmune system, adolescence has emerged as a critical period that may be uniquely sensitive to both acute and long-term ethanol exposure (Crews and Vetreno, 2014). In our laboratory, we previously reported that, when age differences were present, adolescents exhibited blunted neuroimmune responses to either acute ethanol or immune challenge with LPS (Doremus-Fitzwater et al., 2015). A similar reduced response among adolescents to acute ethanol was also reported by Kane et al. (2014). Here, we examined both adolescents and adults with a history of PAE and then assessed their responses to acute binge-like ethanol on brain cytokines. Given the blunted response to acute ethanol in adolescents vs. adults without PAE history, we further anticipated that PAE would engender an even larger blunting to acute ethanol effects on neuroimmune factors among PAE adolescents. While our experimental design did not allow us to directly compare adolescents and adults (as they were two separate cohorts that were analyzed separately), when generally comparing regions and targets examined between adolescents and adults, adolescents mounted immune changes similar in pattern and in magnitude to those exhibited by adults. Thus, these data do not suggest a strong age difference in this RANGE response, as we would have expected. At this time, it is not clear why adolescents in this experiment mounted an ethanol-induced immune response similar to adults. It is possible that this discrepancy may be due to strain differences in developmental

maturity of the neuroimmune system. The present experiments were conducted with Long Evans rats, whereas previous work was done in Sprague–Dawley rats at a slightly earlier age (Doremus-Fitzwater et al., 2015). More work needs to be done to clearly understand the sensitivity of adolescents to PAE and acute ethanol, with particular attention paid to variables such as these.

SUMMARY AND CONCLUSIONS

Overall, the present findings indicate that moderate exposure to gestational alcohol leads to more subtle long-lasting effects on the neuroinflammatory milieu of the offspring. Furthermore, these results would suggest that, for most individuals and at most ages, the primary determinant of the neuroimmune response evoked by a binge-like ethanol challenge is the binge-like challenge, itself. Certainly, additional studies will be required to more fully address this issue. Children with fetal alcohol exposure may not always exhibit obvious or apparent consequences of such exposure, particularly if the prenatal exposure was modest or low in magnitude. However, the evidence is growing to suggest that low to moderate fetal alcohol exposure may cause insidious neuroimmune consequences that are only unmasked when future life events that trigger immune system responses are encountered. Thus, studies such as these are contributing to a growing understanding of how multiple developmental "hits" may enhance or suppress immune system function across ontogeny.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Humane Use of Animals (CHUA) at SUNY-Upstate Medical University (previous employer for SY), and studies were conducted in accordance with the Public Health Service (PHS) policy on the Humane Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

SY and LY prenatally exposed rats to alcohol in SY's research laboratory when previously employed at Upstate Medical University (UMU), and also challenged offspring during adolescence or adulthood with ethanol or vehicle. TD-F, AG, and TD all participated in tissue sample collection at UMU, with TD-F, AG, and AV working to process brain tissue and blood samples in TD's laboratory. TD-F, AV, and TD worked together to analyze data presented in the current manuscript, with TD-F and TD as the primary authors. All authors read, reviewed, and contributed substantially to the manuscript presented here.

FUNDING

This research was supported by the National Institute of Health (National Institute on Alcohol Abuse and Alcoholism) grant P50AA017823 (TD, SY), as well as the Center for Development and Behavioral Neuroscience (CDBN) at Binghamton University. Additional funding support was provided by T32AA025606 and F31AA027959 (AV, TD). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the above stated funding agencies.

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