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EXPERIMENTAL MODELS OF EARLY EXPOSURE TO ALCOHOL: A WAY TO UNRAVEL THE NEUROBIOLOGY OF MENTAL RETARDATION

Topic Editors
Alberto Granato and Andrea De Giorgio

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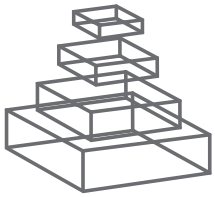

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EXPERIMENTAL MODELS OF EARLY EXPOSURE TO ALCOHOL: A WAY TO UNRAVEL THE NEUROBIOLOGY OF MENTAL RETARDATION

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Excessive alcohol drinking represents a major social and public health problem for several countries. Alcohol abuse during pregnancy leads to a complex syndrome referred to as fetal alcohol spectrum disorders (FASD), chiefly characterized by mental retardation. The effects of early exposure to ethanol can be reproduced in laboratory animals and this helped to answer several key questions concerning the human pathology. The interest of experimental models of FASD is twofold. First, they increase our knowledge about the dose and modality of alcohol consumption able to induce damaging effects on the developing brain. Second, experimental models of FASD can provide useful hints to elucidate the basic mechanisms leading to the intellectual disability. In fact, experimental exposure to alcohol can be carried out during discrete, often very restricted, time windows. As a consequence, FASD models, though depending on the multifaceted interference of alcohol with several molecular pathways, can provide valuable information about which specific developmental periods and brain areas are critically involved in the genesis of mental retardation.

Putting together data obtained through several experimental paradigms of alcohol exposure and those deriving from other genetic and non-genetic models, one can figure out to what extent different types of mental retardation share common pathogenetic mechanisms.

The present Research Topic is aimed at establishing the state of the art of the current research on experimental FASD, focusing on differences and homologies with other types of intellectual disability. The ultimate goal is to find out a common roadmap in view of future therapeutical approaches.

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EDITORIAL
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Experimental models of early exposure to alcohol: a way to unravel the neurobiology of mental retardation

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Keywords: fetal alcohol spectrum disorders, development, cerebral cortex, amygdala, apoptosis, intellectual disability, glial cells, epigenetics

As of November 2014, a PubMed search for "fetal alcohol" retrieved more than 14,500 articles. Alcohol consumption during pregnancy and its detrimental consequences on the developing brain raise major public health, social, and economic issues. However, the research on fetal alcohol spectrum disorders (FASD) in the real world is challenging, given that it is largely based on retrospective analysis. Therefore, establishing the relationship between brain damage and drinking habits proves particularly hard. One of the advantages of FASD studies carried out in the laboratory environment derives from the tight control of time, dose, and modality of alcohol exposure (1). Furthermore, since FASD are among the leading causes of intellectual disability, animal models of early exposure to alcohol represent an invaluable tool to elucidate the basic neurobiological mechanisms leading to the cognitive defects. Experimental models of genetic syndromes are ideally suited to study the role of single molecules, such as the fragile X mental retardation protein, throughout the maturation of the nervous system. Conversely, experimental exposure to alcohol can be carried out during discrete, often very restricted, time windows and, though depending on the interference with several molecular pathways, can provide information about which developmental periods and brain areas are critically involved in the genesis of the intellectual disability.

In the present Research Topic, hosted by Frontiers in Pediatrics, we have gathered some of the most outstanding scientists, among those actively involved in the experimental study of FASD. The reader will be browsing through different subfields of basic research on FASD and we are confident that he/she will get a comprehensive view of the topic, including open questions and useful hints for novel therapeutic interventions.

The review article by Brian Christie and coworkers provides a useful guide for the experimental neurobiologist, highlighting pros and cons of the most widely used animal models of FASD (2). In this paper, there is a particular focus on how to study the behavioral consequences of developmental alcohol exposure. Antonella Peruffo and Bruno Cozzi point out that *in vitro* experiments dealing with neurodegenerative disorders or FASD can be carried out on primary cultures from the fetal bovine brain (3). In view of the concerns raised by the Institutions and by the general public on animal experimentation, species already used for alimentary purposes represent a valuable alternative.

Alcohol can interfere with cell populations that pave the way for brain development. The interaction of ethanol with the pioneering cortical preplate is described in the review by Eric Olson, where a strong focus is devoted to gene expression during early cortical development (4). Neuroscientists sometimes forget the role of glial cells. Thus, we are grateful to Marina Guizzetti and her coworkers, who shed light on how glial cells guide neural development and how this pivotal function can be disrupted during FASD (5).

There is no doubt that the cerebral cortex is one of the key structures affected by early exposure to alcohol and its impairment is responsible for most of the cognitive defects observed in FASD. Alexandre Medina and coworkers contributed to this Research Topic with an original research article in which, combining different methods, they describe deep alterations affecting the visual cortex and the visual pathways of mice exposed to alcohol during early postnatal life (6). Such anomalies can result from the disruption of visual cortical plasticity, demonstrated in previous works from the same lab. In our mini-review in Ref. (7), we focus on neocortical pyramidal neurons and show that FASD and other types of mental retardation are characterized by several, often contrasting, alterations of this heterogeneous neuron population.

Although the cortex and cerebellum are the most studied structures among those damaged by the early effects of alcohol, other brain areas appear to be affected as well. Surprisingly, the amygdala, despite its key role in the emotional and social life, has received little attention. Two original research articles, hosted in this Research Topic, deal with amygdalar alterations in experimental FASD. Using a combination of biochemical, electrophysiological, and behavioral techniques, Fernando Valenzuela and coworkers demonstrated an impairment of dopamine-regulated GABA neurotransmission in the basolateral amygdala (8). Cherry Ignacio, Sandra Mooney, and Frank Middleton (9) studied the micro-RNA expression in the amygdala of rats prenatally exposed to ethanol. It is worth mentioning that the observed alterations were partially reversed by social enrichment. Needless to say, both these papers, focused on the amygdala, display a great potential for the discovery of new therapeutic interventions.

Recently, the gap between genetic and environmental influence has been bridged by the advent of a discipline usually referred to as epigenetics. In their comprehensive review, Shiva Singh and coworkers point out that epigenetic mechanisms (such as those related to DNA methylation) give a substantial contribution to the genesis of the intellectual disability observed in FASD (10).

We guess that the epigenetics of FASD will open new, exciting possibilities for the interpretation of syndromes featuring mental retardation.

Finally, John Olney, in his opinion article (11), raises a challenging issue: even though FASD alterations are manifold, yet all of them can be reconducted to a single starting point, namely the apoptosis. Therefore, most of the research efforts should be concentrated on the treatment of the widespread, ethanol-induced neuronal death. This line of reasoning can be further extended to several conditions, such as the effects of other drugs of abuse and/or of largely used anesthetics.

In conclusion, we wish to thank all the neuroscientists who gave their valuable contribution to this Research Topic. We are confident that their commitment to the experimental work on FASD will ultimately result in a great improvement of our ability to understand the intellectual disability.

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Moderate alcohol exposure during the rat equivalent to the third trimester of human pregnancy alters regulation of $GABA_A$ receptor-mediated synaptic transmission by dopamine in the basolateral amygdala

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Fetal ethanol (EtOH) exposure leads to a range of neurobehavioral alterations, including deficits in emotional processing. The basolateral amygdala (BLA) plays a critical role in modulating emotional processing, in part, via dopamine (DA) regulation of GABA transmission. This BLA modulatory system is acquired during the first 2 weeks of postnatal life in rodents (equivalent to the third trimester of human pregnancy) and we hypothesized that it could be altered by EtOH exposure during this period. We found that exposure of rats to moderate levels of EtOH vapor during the third trimester-equivalent [postnatal days (P) 2–12] alters DA modulation of GABAergic transmission in BLA pyramidal neurons during periadolescence. Specifically, D1R-mediated potentiation of spontaneous inhibitory postsynaptic currents (IPSCs) was significantly attenuated in EtOH-exposed animals. However, this was associated with a compensatory decrease in D3R-mediated suppression of miniature IPSCs. Western blot analysis revealed that these effects were not a result of altered D1R or D3R levels. BLA samples from EtOH-exposed animals also had significantly lower levels of the DA precursor (L-3,4-dihydroxyphenylalanine) but DA levels were not affected. This is likely a consequence of reduced catabolism of DA, as indicated by reduced levels of 3,4-dihydroxyphenylacetic acid and homovanillic acid in the BLA samples. Anxiety-like behavior was not altered in EtOH-exposed animals. This is the first study to demonstrate that the modulatory actions of DA in the BLA are altered by developmental EtOH exposure. Although compensatory adaptations were engaged in our moderate EtOH exposure paradigm, it is possible that these are not able to restore homeostasis and correct anxiety-like behaviors under conditions of heavier EtOH exposure. Therefore, future studies should investigate the potential role of alterations in the modulatory actions of DA in the pathophysiology of fetal alcohol spectrum disorders.

Keywords: fetal, alcohol, BLA, dopamine, GABA, electrophysiology, prenatal, homeostasis

INTRODUCTION

Fetal exposure to ethanol (EtOH) is a leading cause of mental retardation in the world and can lead to a myriad of complications known as Fetal Alcohol Spectrum Disorders (FASDs). FASDs are a major public health problem with an estimated prevalence of 1–5% in the United States (1). FASDs can range from severe mental retardation and facial dysmorphologies to more subtle cognitive/behavioral deficits in the absence of morphological alterations. Importantly, ~20% of children and adolescents with FASDs suffer from emotional processing deficits, such as anxiety, that can manifest into adverse long-term outcomes and poor social adjustment (2). The interventions available to treat emotional processing deficits in patients with FASDs are only partially effective and this is a consequence of our limited understanding of the cellular and molecular underpinnings of these disorders (2).

The amygdala is a key mediator of emotional processing in humans and rodents. The basolateral amygdala (BLA) functions to locally process sensory and cortical information required to generate appropriate emotional responses (3). BLA glutamatergic pyramidal neuron activity is positively correlated with anxiety-like behaviors (4) and excitability of these neurons is regulated by GABAergic interneurons. Stress-inducing novel experiences increase dopamine (DA) release in the BLA (5, 6). In humans, this DA surge has been suggested to regulate amygdala function depending on environmental stimuli (7). In rodents, DA fibers innervate both pyramidal neurons (8) and interneurons (9, 10). Importantly, DA can bi-directionally regulate $GABA_A$ receptor ($GABA_AR$)-dependent synaptic transmission by (1) increasing local interneuron excitability via type-1 DA receptors (D1Rs) (11), and (2) decreasing quantal GABA release onto pyramidal neurons via type-3 receptors (D3Rs) (12). Consistent with the actions of D1Rs in the BLA, it has been demonstrated that microinjection of a D1R antagonist into the BLA results in anxiogenesis (13). In contrast, microinjections of D3R antagonists

directly into the BLA or D3R deficiency reduces anxiety-like behaviors (12, 14).

A common drinking pattern in pregnant women is to abstain from EtOH during the first two trimesters, followed by consumption during the third trimester (15). Low-to-moderate EtOH exposure during late pregnancy has been associated with increased incidence of anxiety disorders in offspring during childhood (16). During the human third trimester of pregnancy, neuronal circuits undergo significant refinement, and a number of neurotransmitter systems are acquired. Similar neuronal processes occur in rodents in the first two postnatal weeks, particularly the development of GABAergic interneurons (17) and DA innervation (18) within the BLA. Exposure to EtOH during this period of rodent development has been used to model human exposure during the third trimester of pregnancy. The objective of this study was to examine whether exposure of rats to moderate EtOH levels during the third trimester-equivalent period impairs D1R- and D3R-dependent modulation of $GABA_AR$-mediated synaptic transmission at pyramidal neurons in the BLA. We also characterized the effect of EtOH on levels of DA, its precursor, and its metabolites in this brain region. Finally, we assessed anxiety-like behaviors in peri-adolescent animals.

MATERIALS AND METHODS

Unless indicated, all drugs and chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

ANIMALS

All animal procedures were approved by the UNM-Health Sciences Center Institutional Animal Care and Use Committee and conformed to NIH Guidelines. Pregnant Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN, USA) and arrived at gestational day 12–16. Dams were individually housed, received food and water *ad libitum*, and had a plastic hut in the cage to reduce stress.

ETHANOL VAPOR CHAMBER EXPOSURE

To model third trimester-equivalent EtOH exposure, we exposed dams with their pups from postnatal day (P) 2 to P12 from 10 a.m. to 2 p.m. daily using vapor inhalation chambers (19, 20). EtOH vapor/air mixture was equilibrated to reach ~3–3.5 g/dL measured with a breathalyzer (Intoximeters, St. Louis, MO, USA). During the 10 days of exposure, animals were handled only once on P5 for bedding and cage change, at which time litters were culled to 8–12 pups. After exposure, offspring were allowed to mature to P40–P50 for electrophysiology experiments, tissue collection, or behavioral testing. Animals were also weighed at P40–P50. Only males were used for this study.

Pup serum EtOH concentrations (SECs) were determined by taking pups immediately after the exposure on P6 and P12. Animals were anesthetized with ketamine (250 mg/kg i.p.), decapitated, and trunk blood was collected and mixed with 6.6% perchloric acid (50 µl of blood and 450 µl of perchloric acid). Samples were centrifuged at a relative centrifugal force of 3000 for 15 min at 4°C. SECs were measured in the supernatants using a standard alcohol dehydrogenase-based assay, as previously described (21).

BLA SLICE ELECTROPHYSIOLOGY

For slice preparation, animals were sacrificed by rapid decapitation under deep anesthesia with ketamine (250 mg/kg i.p.) and brains were quickly removed and submerged for 2 min in cold sucrose artificial cerebro spinal fluid (aCSF) containing (in millimolar): 220 sucrose, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 $MgSO_4$, 10 glucose, 0.2 $CaCl_2$, and 0.43 ketamine, pre-equilibrated with 95% O_2/5% CO_2. Coronal brain slices containing the BLA (250 µm) were prepared using a vibrating tissue slicer (Leica Microsystems, Bannockburn, IL, USA). Immediately following this procedure, slices were placed in a chamber containing normal aCSF and allowed to recover for 40 min at 35–36°C followed by storage at 22°C. Normal aCSF contained (in millimolar): 126 NaCl, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 1 $MgSO_4$, 2 $CaCl_2$, and 0.4 ascorbic acid and was continuously equilibrated with 95% O_2/5% CO_2.

For whole-cell patch-clamp electrophysiological recordings, neurons were visualized using infrared-differential interference contrast microscopy and recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). BLA pyramidal neurons were identified on the basis of their morphology (large/pyramidal-shaped) and capacitance ≥150 pF. To record spontaneous inhibitory postsynaptic currents (sIPSCs), a KCl-based internal solution was used (12). In some experiments, tetrodotoxin (TTX; 1 µM; Tocris, Ellisville, MO, USA) was added to the aCSF to block action potential-dependent events and record miniature IPSCs (mIPSCs). IPSCs were isolated by blocking AMPA and NMDA receptors using kynurenic acid (1 mM) and DL-APV (50 µM; Tocris). The holding potential was −70 mV. During application of glutamate antagonists, neurons were allowed to equilibrate for at least 5 min prior to beginning an experiment. Data were acquired in gap-free mode at 10 kHz and filtered at 2 kHz. Only recordings where the access resistance changed <20% were kept for analysis.

WESTERN IMMUNOBLOTTING

BLA tissue was micro-dissected from coronal slices from Air and EtOH animals. These were prepared as described above, collected immediately after slicing, and flash frozen in liquid nitrogen. Tissue was then sonicated in homogenization buffer (0.1 g tissue/1 mL of buffer) containing: 25 mM HEPES (pH 7.4), 500 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1% Tween-20, 1 mM phenylmethanesulfonyl fluoride, 20 mM NaF, 1% v/v phosphatase cocktail (Sigma-Aldrich, Cat # P2850), 5 µM cyclosporine A, and 1 Complete Mini Protease tablet/10 mL (Cat # 11836153001, Roche Diagnostics, Indianapolis, IN, USA), and stored in 10 µL aliquots at −80°C. The protein concentration was determined by the Bradford Method (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard.

Samples were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer (Final concentration: 250 mM Tris–HCl (pH 6.8), 10% sodium dodecyl sulfate, 30% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue), and boiled at 95°C for 5 min. Samples were loaded at a concentration of 10 µg per lane. Control experiments demonstrated that this concentration of protein was within the linear dynamic range

for the western blot assay (not shown). Electrophoresis was performed in 4–15% Tris–HCl precast gels (BioRad) at 140 V for 60 min at 4°C. Proteins were blotted onto polyvinylidene fluoride membranes (0.4 μm pore size) at 100 V for 60 min at 4°C. Non-specific binding was blocked with Odyssey Blocking Buffer (Li-Cor, Lincoln, NE, USA) for 1 h at room temperature and probed overnight at 4°C with either of the following specific primary antibodies: anti-D1R antibody (1:1000, Cat # 20066; Abcam, Cambridge, England) or anti-D3R antibody (1:500, Cat # AB1786P, Millipore, Temecula, CA, USA), and mouse anti-β-actin monoclonal antibody (1:50000, Sigma-Aldrich). Visualization and relative protein densities were quantified using an Infrared Imaging System (Odyssey, LI-COR System). Membranes were then incubated for 20 s in 0.025% (w/v) Coomassie Blue R-250, 40% methanol, and 7% acetic acid in water and washed overnight with a de-staining solution containing 50% methanol and 10% acetic acid. Membranes were scanned and Coomassie staining quantified using ImageJ 1.46r software (22). For each sample, the protein expression was normalized to β-actin or an average of the intensity of randomly selected Coomassie-stained bands.

TISSUE CONTENT OF DA AND RELATED ANALYTES

BLA-containing slices were prepared as the western immunoblotting experiments and the BLA was immediately micro-dissected and snap-frozen in liquid nitrogen. Samples were homogenized in 250 μL of 0.1 M $HClO_4$ and the protein concentration was determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA). Extracts were centrifuged and supernatants were removed and analyzed for DA, its precursor L-3,4-dihydroxyphenylalanine (L-DOPA), and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using high performance liquid chromatography (HPLC) coupled to electrochemical detection at +220 mV (ESA Inc., Chelmsford, MA, USA). Analytes were separated on a Luna 100 mm × 3.0 mm C_{18} 3 μm reverse phase HPLC column (Phenomenex, Torrance, CA, USA). The mobile phase for L-DOPA consisted of 10 μM NaCl, 46 mM NaH_2PO_4, 172 μM sodium octyl sulfate, 100 μM EDTA, 10% methanol, pH to 2.6 before adding the methanol and sodium octyl sulfate. For the other analytes, the mobile phase consisted of 50 mM citric acid, 90 mM NaH_2PO_4, 1.7–2.0 mM 1-octanesulfonic acid, 50 μM EDTA, 10–12% C_2H_3N, and 0.3% triethylamine (pH 3.0). Analytes were quantified using PowerChrom software (eDAQ Inc, Colorado Springs, CO, USA) and a standard calibration curve.

ELEVATED PLUS MAZE

Anxiety-like behavior was assessed using an elevated plus-maze apparatus similar to that originally described (23) with incandescent lighting (~13 lux at junction). The maze consisted of two open arms and two closed arms (50.8 cm long in all cases). The open arms had a ledge 1 cm high to prevent the animals from slipping off the edge. The closed arms were surrounded by walls 30.5 cm tall. The plus-maze platform was elevated 50.8 cm above the floor. Animals were allowed to acclimate to ambient lighting and noise in the testing room for 1 h. During testing, animals were allowed to freely move in the maze for 5 min. After testing, animals were not returned to the home cage until all animals from a cage were tested to prevent exposure to stress from the tested animal (24). Between animals, the apparatus was cleaned with 70% EtOH and thoroughly dried. Animal activity was video recorded, and time spent in the open arms, closed arms, and junction, and the number of times an animal engaged in a stretch attend posture were recorded by an investigator blinded to the experimental condition of the subject.

DATA ANALYSES

Electrophysiology recordings were initially analyzed with Mini-Analysis (Synaptosoft, Decatur, GA, USA). All data were statistically analyzed with Prizm 5 (Graphpad, San Diego, CA, USA). Initially, data were analyzed with the Pearson omnibus normality test. Only data that followed a normal distribution were analyzed using parametric tests. A $p < 0.05$ was considered to be statistically significant. Unless indicated, the experimental unit used for all statistical analyses was an animal (i.e., results obtained with all the slices from a single animal were averaged to yield a unit of determination).

RESULTS

CHARACTERIZATION OF THIRD TRIMESTER-EQUIVALENT ETHANOL VAPOR MODEL

To model EtOH exposure during the third trimester-equivalent, dams and pups were exposed to vaporized EtOH for 4 h per day from P2 to P12. This exposure resulted in pup SECs of 22.7 ± 1.4 mM ($n = 16$; as a reference, the legal intoxication limit in the U.S. is 17.4 mM = 0.08 g/dL); this paradigm attempted to model human exposure to relatively moderate levels of EtOH (25). There were no significant differences in pup weight between the Air and EtOH groups at the time of the electrophysiology experiments (average pup weight at P40-P50: Air = 166.6 ± 5.74 g, $n = 8$; EtOH = 158.2 ± 7.12 g, $n = 11$; Mann–Whitney $U = 36.50$, sum of ranks = 87.50, 102.5; $p > 0.05$ by Mann–Whitney test).

ETHANOL EXPOSURE DID NOT AFFECT BASAL GABAergic TRANSMISSION IN PYRAMIDAL NEURONS

Whole-cell patch-clamp electrophysiological recordings revealed that EtOH exposure had no effect on pyramidal neuron membrane capacitance (Air = 231.50 ± 19.49 pF, $n = 10$; EtOH = 222.10 ± 8.97 pF, $n = 10$; $t = 0.44$, df = 18, $p > 0.05$ by unpaired t-test) or membrane resistance (Air = 140.90 ± 30.05 MΩ, $n = 10$; EtOH = 123.40 ± 10.77 MΩ, $n = 10$; Mann–Whitney $U = 46$, sum of ranks = 109, 101, $p > 0.05$ by Mann–Whitney test). There was also no effect of EtOH exposure on basal sIPSC frequency (**Figures 1A,B**; $n = 8$, $t = 0.63$, df = 14, $p > 0.05$ by unpaired t-test) or amplitude (**Figures 1A,C**; $n = 8$, Mann–Whitney $U = 30$, sum of ranks = 66, 70, $p > 0.05$ by Mann–Whitney test). Similarly, EtOH exposure did not alter basal mIPSC frequency (**Figures 1D,E**; $n = 8$, $t = 1.84$, df = 14, $p > 0.05$ by unpaired t-test) or amplitude (**Figures 1D,F**; $n = 8$, $t = 0.07$, df = 14, $p > 0.05$ by unpaired t-test). In a different subset of pyramidal neurons from Air-exposed animals, we measured the effect of TTX on GABAergic transmission and found that TTX did not significantly alter sIPSC frequency (TTX-induced inhibition: 14.93 ± 9.56%, $n = 7$, $t = 1.56$,

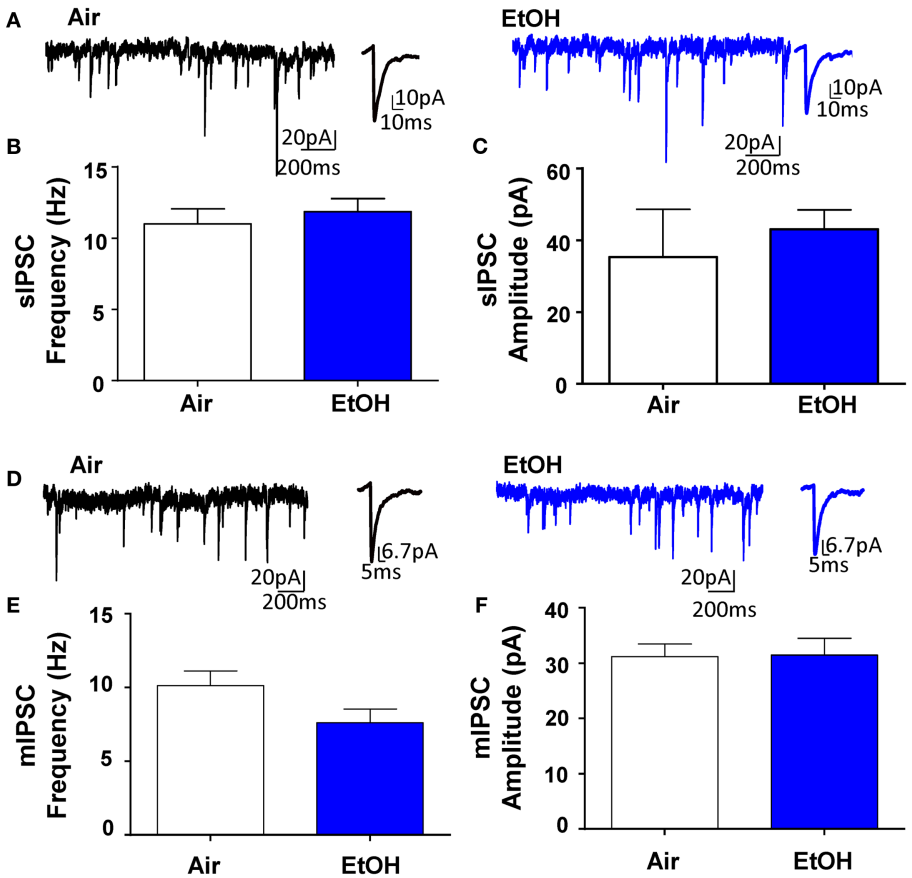

FIGURE 1 | Ethanol exposure does not affect basal spontaneous inhibitory postsynaptic currents (sIPSCs) or miniature IPSCs (mIPSCs) in the BLA. **(A)** Exemplar compressed traces of sIPSC recordings obtained under baseline conditions from Air (black) and EtOH (blue) exposed animals. Exemplar expanded scale traces of averaged sIPSCs are shown adjacent to compressed traces. Basal sIPSC **(B)** frequency and **(C)** amplitude were not significantly different between Air- and EtOH-exposed animals ($n = 8$ animals from 5 Air and 5 EtOH litters, $p > 0.05$ by unpaired t-test). **(D)** Exemplar compressed traces of mIPSC recordings obtained under baseline conditions (in the presence of 1 μM TTX) from Air (black) and EtOH (blue) exposed animals. Exemplar expanded scale traces of averaged mIPSCs are shown adjacent to compressed traces. Basal mIPSC **(E)** frequency and **(F)** amplitude were not significantly different between Air- and EtOH-exposed animals ($n = 8$ animals from 5 Air and 6 EtOH litters, $p > 0.05$ by unpaired t-test).

df = 6, $p > 0.05$ by one sample t-test compared to 0), but did significantly decrease sIPSC amplitude (TTX-induced inhibition: 16.30 ± 2.88%, $n = 7$, $t = 5.66$, df = 6, $p < 0.01$ by one sample t-test compared to 0). We confirmed that all events were blocked by the $GABA_AR$ antagonist, gabazine ($n = 5$; 50 μM, Tocris).

ETHANOL EXPOSURE ATTENUATED D1R-MEDIATED POTENTIATION OF sIPSCs IN PYRAMIDAL NEURONS

In agreement with the literature (11, 26), application of DA (50 μM) significantly increased sIPSC frequency in Air-treated animals (**Figures 2A,C**; $n = 8$, $t = 5.15$, df = 7, $p < 0.01$ by one sample t-test compared to 0). In EtOH-treated animals DA also significantly increased sIPSC frequency (**Figures 2B,C**; $n = 8$, $t = 4.78$, df = 7, $p < 0.01$ by one sample t-test compared to 0); however, this effect was significantly blunted compared to Air-exposed animals (**Figure 2C**; $t = 2.73$, df = 14, $n = 8$, $p < 0.05$ by unpaired t-test). Application of DA (50 μM) significantly increased sIPSC amplitude in Air-treated animals (**Figures 2A,D**; $n = 8$, $t = 2.71$, df = 7, $p < 0.05$ by one sample t-test compared to 0). In contrast, the sIPSC amplitude was not altered by DA in the EtOH-exposed slices (**Figures 2B,D**; $n = 8$, $t = 1.61$, df = 7, $p > 0.05$ by one sample t-test compared to 0). The DA-induced effect on sIPSC amplitude was significantly different between Air- and EtOH-treated animals (**Figure 2D**; Mann–Whitney $U = 8$, sum of ranks = 92, 44, $n = 8$, $p < 0.05$ by Mann–Whitney test).

The DA-induced increase of sIPSC frequency has been shown to be mediated by activation of D1Rs, which increase the excitability of local interneurons (11, 26, 27). To confirm these findings, we first examined the effect of the D1R antagonist, SCH23390 (10 μM), on GABAergic transmission from Air-exposed animals and found that this agent alone did not induce a significant change in basal sIPSC frequency (change from baseline: 12.28 ± 8.70%; $n = 5$, $t = 1.41$, df = 4, $p > 0.05$ compared to 0 by one sample t-test) or amplitude (change from baseline: 13.96 ± 13.06%; $n = 5$, $t = 1.07$, df = 4, $p > 0.05$ compared to 0 by one sample t-test). SCH23390 also did not alter mIPSC frequency (change from baseline: −5.37 ± 6.24%; $n = 5$, $t = 0.86$, df = 4, $p > 0.05$ compared

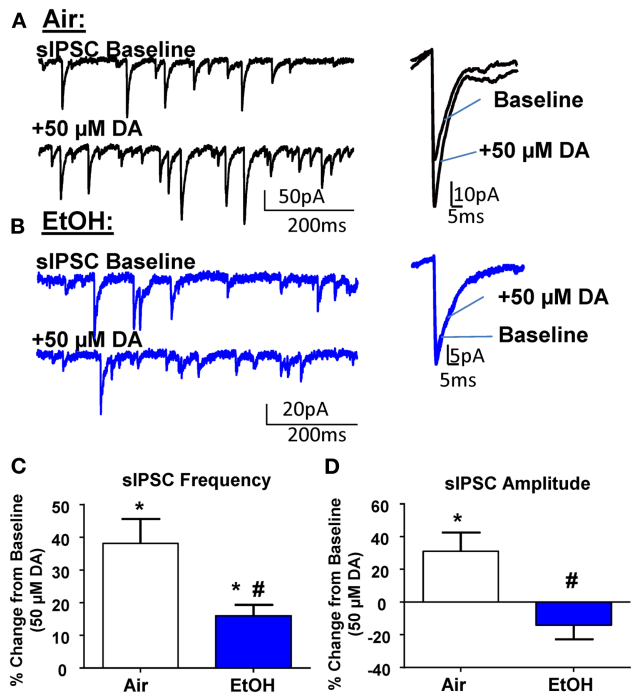

FIGURE 2 | Ethanol exposure impairs D1R-mediated facilitation of $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) in the BLA. Exemplar compressed traces of sIPSC recordings obtained under baseline conditions and in the presence of 50 μM dopamine (DA) in slices from **(A)** Air- and **(B)** EtOH-exposed animals. Exemplar expanded scale traces of averaged sIPSCs are shown adjacent to compressed traces. **(C)** 50 μM DA significantly increased sIPSC frequency in Air animals ($^*p < 0.01$ by one sample *t*-test compared to 0) and EtOH animals ($^*p < 0.01$ by one sample *t*-test compared to 0). The DA-induced increase in sIPSC frequency was significantly attenuated in EtOH compared to Air-exposed animals ($^\#p < 0.05$ by unpaired *t*-test). **(D)** sIPSC amplitude was significantly increased by 50 μM DA in Air ($^*p < 0.05$ by one sample *t*-test compared to 0), but not EtOH-exposed animals ($p > 0.05$ by one sample *t*-test compared to 0). The DA-induced potentiation of sIPSC amplitude was significantly blunted in the EtOH compared to Air-exposed animals ($^\#p < 0.05$ by Mann–Whitney test). $N = 8$ animals from 8 Air and 7 EtOH litters.

to 0 by one sample *t*-test) or amplitude (change from baseline: $-12.12 \pm 11.13\%$; $n = 5$, $t = 1.08$, $df = 4$, $p > 0.05$ compared to 0 by one sample *t*-test). In the presence of SCH23390, DA did not significantly change sIPSC frequency in either Air-exposed (change from baseline: $-14.46 \pm 8.57\%$; $n = 8$, $t = 1.69$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test) or EtOH-exposed (change from baseline: $2.12 \pm 2.84\%$; $n = 8$, $t = 0.74$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test). Similarly, in the presence of SCH23390, DA did not significantly change sIPSC amplitude in Air (change from baseline: $-0.87 \pm 10.48\%$; $n = 8$, $t = 0.08$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test) or EtOH-treated animals (change from baseline: $-13.29 \pm 9.78\%$; $n = 8$, $t = 1.36$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test).

It is important to note that micromolar concentrations of DA have been shown to activate noradrenergic receptors, specifically α1 (28). Furthermore, norepinephrine-mediated activation of α1-adrenoreceptors in the BLA can increase GABA transmission (29), similar to the D1R-mediated effect on sIPSCs. Therefore, we investigated the effect of DA (50 μM) on sIPSCs in the presence of the selective α1 antagonist, doxazosin (25 μM; Tocris), and found that DA still significantly increased sIPSC frequency (change from baseline: $27.58 \pm 5.19\%$; $n = 6$, $t = 5.31$, $df = 5$, $p < 0.05$ compared to 0 by one sample *t*-test) without altering sIPSC amplitude (change from baseline: $-12.90 \pm 11.07\%$; $n = 6$, $t = 1.16$, $df = 5$, $p < 0.05$ compared to 0 by one sample *t*-test). Importantly, there was no significant difference between the effect of DA and DA + doxazosin on sIPSC frequency ($t = 1.09$, $df = 12$, $p > 0.05$ by unpaired *t*-test). However, there was a significant difference between the effect of DA ($n = 8$) and DA + doxazosin on sIPSC amplitude ($n = 6$, Mann–Whitney $U = 7$, sum of ranks = 77, 28, $p < 0.05$ by Mann–Whitney test).

EXPOSURE TO ETHANOL IMPAIRED D3R-MEDIATED SUPPRESSION OF mIPSCs IN PYRAMIDAL NEURONS

Application of DA (50 μM) significantly decreased mIPSC frequency in Air-treated animals (**Figures 3A,C**; $n = 8$, $t = 3.44$, $df = 7$, $p < 0.05$ compared to 0 by one sample *t*-test). A similar effect had previously been shown using a selective D3R agonist, which can reduce quantal GABA release from local interneurons (12). Interestingly, in EtOH-treated animals, DA did not significantly alter mIPSC frequency (**Figures 3B,C**; $n = 8$, $t = 0.31$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test); and this effect was significantly different compared to Air-exposed animals (**Figure 3C**; $t = 2.67$, $df = 14$, $n = 8$, $p < 0.05$ by unpaired *t*-test). Application of DA (50 μM) did not significantly alter mIPSC amplitude in either Air- (**Figure 3D**; $t = 0.27$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test) or EtOH-treated animals (**Figure 3D**; $t = 1.52$, $df = 7$, $n = 8$, $p > 0.05$ compared to 0 by one sample *t*-test) and this effect of DA on mIPSC amplitude was not significantly different between Air- and EtOH-treated slices (**Figure 3D**; $t = 0.67$, $df = 14$, $n = 8$, $p > 0.05$ by unpaired *t*-test).

To determine if there was a tonic D3R activation, we first examined the effect of the selective D3R antagonist, GR103691 (1 μM; Tocris), on GABA transmission. After statistically identifying and removing one outlier (Grubbs outlier test), we found that GR103691 alone did not alter sIPSC frequency (change from baseline: 16.36 ± 14.61; $n = 5$, $t = 1.12$, $df = 4$, $p > 0.05$ compared to 0 by one sample *t*-test) or amplitude (change from baseline: $-13.40 \pm 10.98\%$; $n = 6$, $t = 1.22$, $df = 4$, $p > 0.05$ compared to 0 by one sample *t*-test). Likewise, GR103691 alone did not affect mIPSC frequency (change from baseline: -3.35 ± 4.04; $n = 5$, $t = 0.82$, $df = 4$, $p > 0.05$ compared to 0 by one sample *t*-test) or amplitude (change from baseline: $6.27 \pm 7.44\%$; $n = 5$, $t = 0.84$, $df = 4$, $p > 0.05$ compared to 0 by one sample *t*-test). Consistent with previous reports (12), in the presence of GR103691 (1 μM), DA did not significantly change mIPSC frequency (DA-induced change: $10.38 \pm 6.05\%$, $n = 8$, $t = 1.72$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test) or amplitude (DA-induced change: $-0.76 \pm 4.19\%$, $n = 8$, $t = 0.18$, $df = 7$, $p > 0.05$ compared to 0 by one sample *t*-test) in Air-exposed animals.

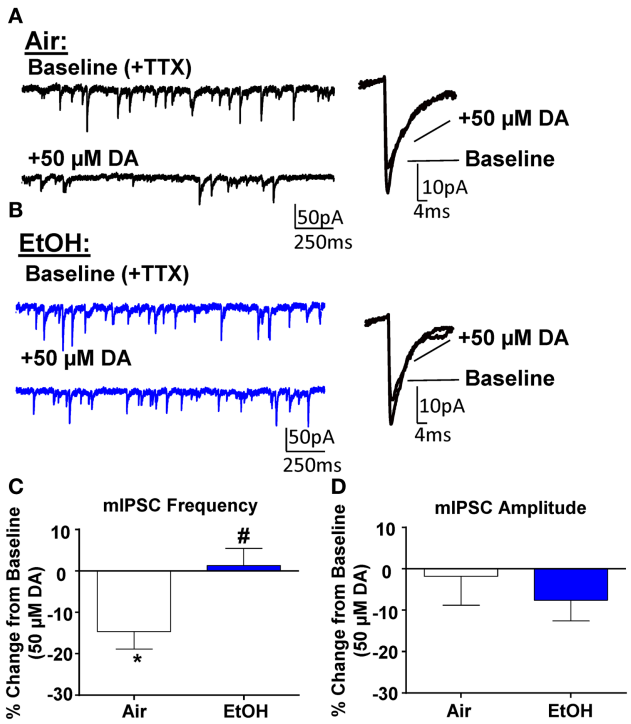

FIGURE 3 | Ethanol exposure impairs D3R-mediated suppression of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) in the BLA. Exemplar compressed traces of mIPSC recordings obtained under Baseline conditions (in the presence of 1 μM TTX) and in the presence of 50 μM dopamine (DA) in slices from **(A)** Air- and **(B)** EtOH-exposed animals. Exemplar expanded scale traces of averaged mIPSCs are shown adjacent to these. **(C)** 50 μM DA significantly decreased mIPSC frequency in Air animals ($^*p < 0.05$ by one sample *t*-test compared to 0), but not in EtOH animals ($p > 0.05$ by one sample *t*-test compared to 0). The DA-induced decrease in mIPSC frequency was significantly attenuated in EtOH compared to Air-exposed animals ($^\#p < 0.05$ by unpaired *t*-test). **(D)** mIPSC amplitude was not altered by 50 μM DA in either Air or EtOH-exposed animals. $N = 8$ animals from 6 Air and 5 EtOH litters.

D1R AND D3R EXPRESSION WAS NOT AFFECTED BY ETHANOL EXPOSURE

The EtOH-mediated effects on D1R and D3R function could be explained by a decrease in the levels of these receptors. Western blot analysis of total D1R expression within the BLA (**Figure 4A**) showed no effect of EtOH exposure when normalized to either Coomassie-stained bands (**Figure 4B**; Mann–Whitney $U = 28$, sum of ranks = 64, 72, $n = 8$, $p > 0.05$ by Mann–Whitney test) or β-actin (**Figure 4C**; $t = 0.13$, df = 14, $n = 8$, $p > 0.05$ by unpaired *t*-test). Similarly, D3R expression within the BLA (**Figure 4D**) was not significantly altered by EtOH exposure when normalized to Coomassie-stained bands (**Figure 4E**; $t = 0.04$, df = 14, $n = 8$, $p > 0.05$ by unpaired *t*-test) or β-actin (**Figure 4F**; $t = 0.18$, df = 14, $n = 8$, $p > 0.05$ by unpaired *t*-test).

EXPOSURE TO ETHANOL DECREASED DA METABOLITE LEVELS IN THE BLA

Several studies have shown that developmental exposure to EtOH leads to decreased DA levels throughout the brain (30). Therefore, we measured levels of DA, its precursor, and its metabolites from micro-dissected BLA samples (**Figure 5A**). We found that EtOH-exposed animals had significantly reduced levels of the DA precursor, L-DOPA (**Figure 5B**; $n = 8$, $t = 2.92$, df = 14, $p < 0.05$ by unpaired *t*-test). However, DA levels were unaltered by EtOH exposure (**Figure 5C**; $n = 8$, $t = 0.54$, df = 14, $p > 0.05$ by unpaired *t*-test), This is probably a consequence of reduced degradation of DA; levels of its metabolites were significantly reduced in the samples from the EtOH group (DOPAC **Figure 5D**; $n = 8$, Mann–Whitney $U = 11$, sum of ranks = 89, 47, $p < 0.05$ by Mann–Whitney test; HVA; **Figure 5E**; $n = 8$, Mann–Whitney $U = 10$, sum of ranks = 90, 46, $p < 0.05$ by Mann–Whitney test).

ANXIETY-LIKE BEHAVIOR WAS NOT AFFECTED BY ETHANOL EXPOSURE IN PERIADOLESCENCE

We next examined anxiety-like behavior using the elevated plus maze. We did not find any changes in the time spent in the open arms, a reliable measure of anxiety-like behavior (**Figure 6A**; $n = 8$ Air and 11 EtOH, $t = 0.29$, df = 17, $p > 0.05$ by unpaired *t*-test). Likewise, there was no difference in the time spent in the closed arms (**Figure 6B**; $n = 8$ Air and 11 EtOH, $t = 0.29$, df = 17, $p > 0.05$ by unpaired *t*-test), or time spent at the arm junction (**Figure 6C**; $n = 8$ Air and 11 EtOH, $t = 0.97$, df = 17, $p > 0.05$ by unpaired *t*-test), suggesting no difference in locomotion. Finally, we also assessed the number of times an animal engaged in a stretch attend posture (a measure of risk assessment) and found no significant differences between Air- and EtOH-exposed animals (**Figure 6D**; $n = 8$ Air and 11 EtOH, $t = 0.43$, df = 17, $p > 0.05$ by unpaired *t*-test).

DISCUSSION

This is the first characterization of the effects of moderate EtOH exposure during the third trimester-equivalent on the modulatory actions of DA in the BLA. We found that EtOH exposure significantly reduced the D1R-mediated enhancement of action potential-dependent spontaneous $GABA_A$R-mediated transmission in pyramidal neurons. We demonstrated that EtOH exposure reduced D3R-mediated reduction of quantal GABA release at interneuron-to-pyramidal neuron synapses, which may represent a compensatory change aimed at restoring balance in the modulatory actions of DA in the BLA. We also found that EtOH exposure induced a decrease in the levels of the DA precursor, L-DOPA. However, DA levels were not significantly affected by EtOH and our findings suggest that this is a consequence of reduced degradation of DA. A model depicting these changes is shown in **Figure 7**. Importantly, anxiety-like behavior on the elevated plus maze was unaffected in EtOH-exposed rats, indicating that homeostasis was re-established in the BLA of treated animals.

MODERATE POSTNATAL ETHANOL EXPOSURE REDUCES THE D1R-MEDIATED ENHANCEMENT OF sIPSC FREQUENCY AND AMPLITUDE

In agreement with the literature (11, 26), D1R activation increased the frequency of sIPSCs in BLA pyramidal neurons from Air-exposed animals. This effect has been shown to be mediated by increased interneuron firing (11). We also found a DA-mediated increase in sIPSC amplitude that was blocked by the D1R

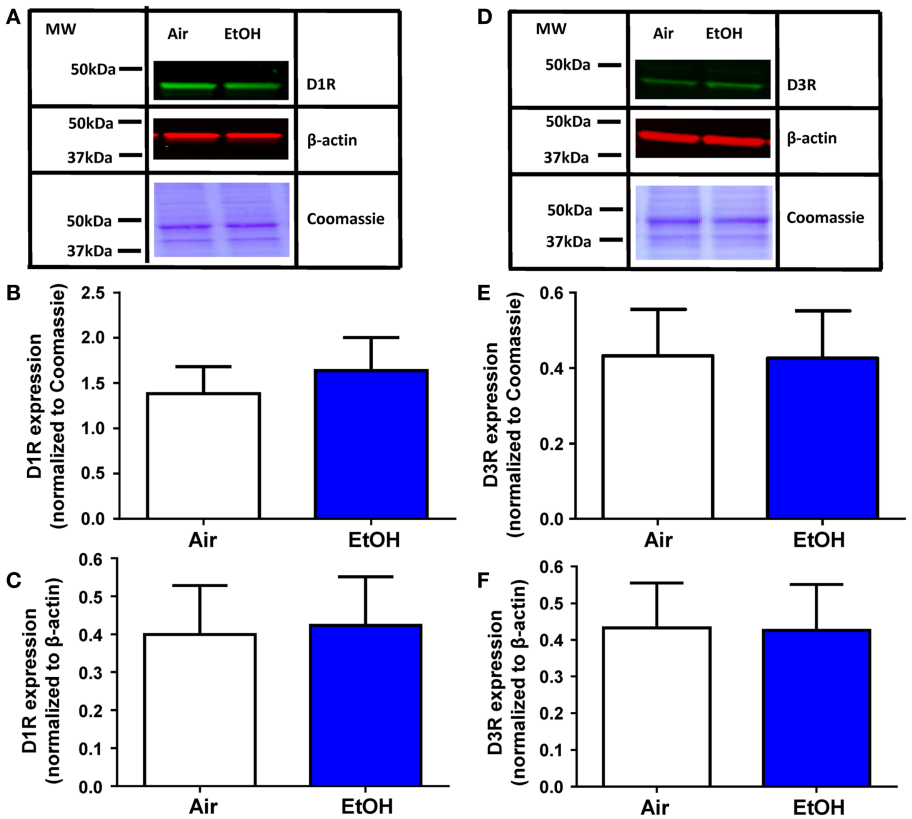

FIGURE 4 | D1R and D3R expression in the BLA is not affected by ethanol exposure. **(A)** Exemplar western blots illustrating levels of D1R and β-actin levels in samples from Air- and EtOH-exposed rats. Also shown are Coommassie stained bands. Graphic quantification of D1R expression normalized to **(B)** Coomassie-stained bands and **(C)** β-actin. D1R expression was not altered by EtOH exposure in either analysis. **(D)** Same as in **(A)** but for D3R. Graphic quantification of D3R expression normalized to **(E)** Coomassie-stained bands and **(F)** β-actin. D3R expression was not altered by EtOH exposure in either analysis. $N = 8$ animals from 4 Air and 4 EtOH litters for all western blots.

antagonist that had not been previously reported. However, studies have either used a lower concentration of DA (11) or did not report sIPSC amplitude (26). It is possible that D1R activation could increase the duration of action potentials in interneurons, leading to an increase in sIPSC amplitudes in the pyramidal neurons (31, 32). Postsynaptic D1Rs (8, 11) may also directly potentiate $GABA_A$Rs, although DA has been shown to not alter exogenous GABA-induced currents in mice (33). Another possibility is that at this concentration, DA may activate noradrenergic receptors, specifically α1 (28), which have been shown to increase GABA transmission in the BLA (29). Consistent with this, the α1 antagonist blocked the effect of DA on sIPSC amplitude, but not on sIPSC frequency. As previously mentioned, the D1R antagonist, SCH23390, also blocked the DA-induced increase in sIPSC amplitude. A likely explanation for this is that SCH23390 is a known blocker of inward rectifier K^+ channels (34–36) and activation of α1-adrenoreceptors by DA can inhibit these channels (28). These data suggest that the effect of DA on sIPSC amplitude may be mediated by α1-adrenoreceptors. Future studies should examine interactions between the DA and noradrenergic systems in the BLA.

Importantly, the DA-mediated facilitation of GABA transmission was significantly blunted in peri-adolescent animals that were exposed to EtOH during the third trimester-equivalent. These alterations cannot be explained by changes in basal properties of sIPSCs as EtOH did not affect basal GABA transmission. Although western blot analysis indicated that total D1R levels within the BLA are not affected by EtOH exposure, a selective decrease in expression of D1Rs in local BLA interneurons could explain the change in D1R function and this should be further investigated. Other potential mechanisms that may result in decreased D1R function in the BLA include: (1) uncoupling of D1Rs from G proteins by a phosphorylation-dependent mechanism (37), (2) internalization of D1Rs (38), or (3) disrupted D1R-mediated signal transduction (37). It is worth noting that the small effect of TTX on sIPSC frequency (**Figure 1**) suggests that D1R activation engages a population of inputs that are normally silent under basal conditions. DA-positive terminals have been found to innervate parvalbumin- and calretinin-positive interneurons (10). Therefore, it is possible that under certain conditions where D1R activation is required to enhance GABAergic inhibition, DA release targets specific interneuron populations in the BLA. Future studies are necessary to determine the mechanism of action of EtOH on D1Rs, and potentially α1-adrenoreceptors, in the BLA.

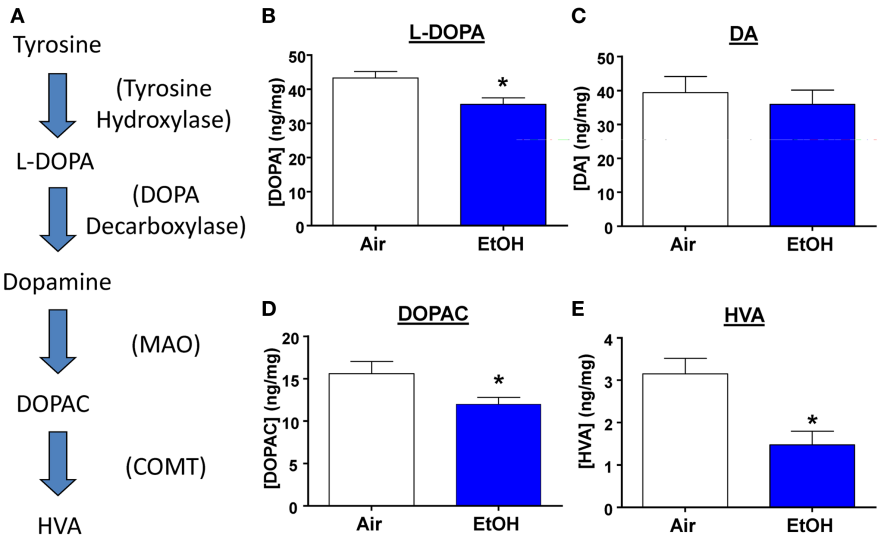

FIGURE 5 | L-DOPA and DA metabolite levels are decreased by ethanol exposure. **(A)** Schematic representation of the DA synthesis pathway. Ethanol exposure significantly reduced levels of **(B)** L-DOPA (*$p < 0.05$ by unpaired *t*-test), but did not alter **(C)** DA levels ($p > 0.05$ by unpaired *t*-test). EtOH exposure also significantly reduced levels of **(D)** DOPAC (*$p < 0.05$ by Mann–Whitney test), and **(E)** HVA (*$p < 0.05$ by Mann–Whitney test). $N = 8$ animals from 4 Air and 4 EtOH litters.

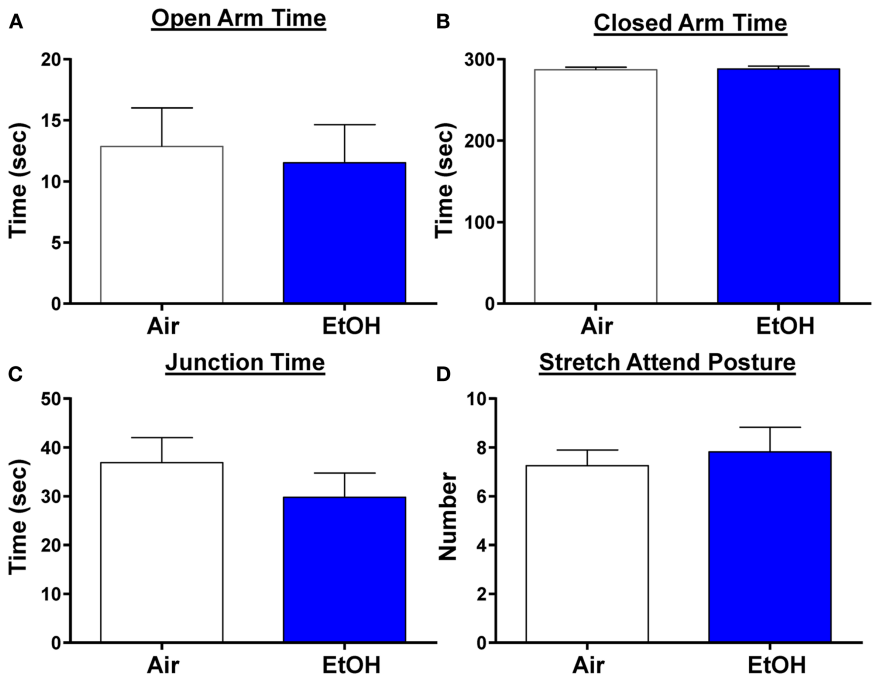

FIGURE 6 | Ethanol exposure did not alter anxiety-like behavior on the elevated plus maze. Exposure to ethanol had no effect on **(A)** open arm time ($p > 0.05$ by unpaired *t*-test), **(B)** closed arm time ($p > 0.05$ by unpaired *t*-test), **(C)** junction time ($p > 0.05$ by unpaired *t*-test), or **(D)** number of times animals engaged in stretch attend posture ($p > 0.05$ by unpaired *t*-test). $N = 8$ animals from 3 Air litters and 11 animals from 5 EtOH litters.

MODERATE POSTNATAL ETHANOL EXPOSURE ABOLISHES THE D3R-MEDIATED DECREASE OF mIPSC FREQUENCY

It has been shown that a selective D3R agonist inhibits quantal GABA release from local interneurons onto BLA pyramidal neurons (12), and we were able to replicate these findings using exogenous DA. Although the mechanism of this presynaptic effect is unclear, D3R activation can suppress extracellular signal-regulated kinase (ERK) activity (39), which can result in decreased

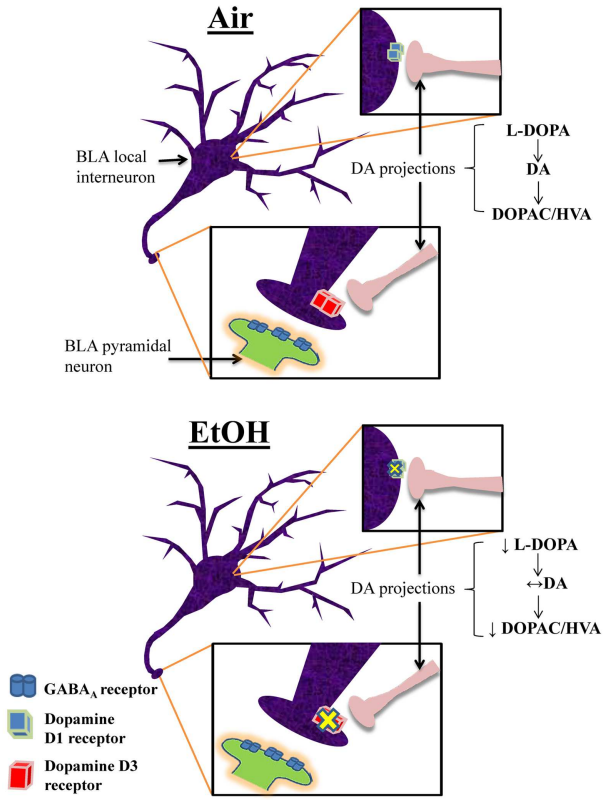

FIGURE 7 | Proposed model of the effects of third trimester-equivalent ethanol exposure on dopamine function in the BLA. The top panel illustrates a local GABAergic interneuron inhibiting a pyramidal neuron in the BLA (postsynaptic pyramidal neuron dendrite only shown in zoomed in image). DA projections from the ventral tegmental area innervate local interneurons, releasing DA onto D1Rs and D3Rs (the exact location of DA innervation onto these local interneurons is unknown). In Air-exposed animals, D1R activation increases interneuron firing, resulting in increased action potential-dependent spontaneous GABA release. Conversely, D3R activation suppresses quantal action potential-independent release of GABA from interneurons. These two opposing effects regulate the balance of GABA transmission in pyramidal neurons. In EtOH-exposed animals, D1R and D3R function is significantly blunted, without changes in receptor levels. Furthermore, although L-DOPA levels are robustly reduced, DA levels remain unchanged due to decreased degradation of DA into DOPAC and HVA. These homeostatic changes presumably explain the normal behavior observed in the elevated plus maze.

GABA release (40). It is worth noting that a postsynaptic D3R effect (i.e., a reduction in mIPSC amplitude) was previously reported using the D3R agonist (12). It is possible that this postsynaptic D3R-mediated effect was masked by either a D1R-dependent or a potential α1-adrenoreceptor-mediated potentiation given that those receptors were not blocked in this set of experiments. However, given that the D3R effect on mIPSC amplitude was similar between the treatment groups, our findings suggest that postsynaptic receptors were not altered by the EtOH exposure.

Interestingly, the D3R-mediated suppression of GABA transmission was completely abolished in EtOH-exposed animals. Together with a deficit in D1R-mediated enhancement of GABA transmission, these findings suggest that homeostatic changes occurred in the DA system. Specifically, EtOH exposure may have decreased function of one of these DA receptors, and the system decreased function in another DA receptor that exerts an opposite effect on GABA release, perhaps as a compensatory mechanism to maintain stable GABAergic inhibition. Based on our findings, it is difficult to conclude which was first impaired by EtOH exposure. However, a human study reported that infants exposed to EtOH through the third trimester showed reduced alert and attentive states during testing (41), which may be an indicator of suppressed amygdala activation. Fetal EtOH-exposed infants exhibited slower registration of auditory and visual stimuli (42), indicating deficits in sensory processing, which may contribute to impaired behavioral arousal. Moreover, a recent study demonstrated that fetal EtOH exposure was robustly associated with higher infant emotional withdrawal (43), which can lead to altered amygdala development (44). Deficits in D3R function in the BLA would result in over-inhibition of this brain region and could explain these behavioral alterations, suggesting that D3Rs could be a primary target of EtOH early in development. Additionally, studies are required to further understand the effects of third trimester-equivalent EtOH exposure on the development of the DA system in the BLA.

Western blot analysis also showed that total D3R expression in the BLA was unaltered by EtOH exposure. However, mechanisms similar to those described for alterations in D1R function may explain the loss of D3R function: (1) uncoupling of D3Rs from G proteins by a phosphorylation-dependent mechanism (45–47), (2) internalization of D3Rs (38), or (3) interrupted D3R-mediated signal transduction (48). These possible explanations require further investigation.

MODERATE POSTNATAL ETHANOL EXPOSURE REDUCES THE LEVELS OF L-DOPA, DOPAC, AND HVA

DA synthesis is initiated by conversion of tyrosine to L-DOPA by tyrosine hydroxylase, which is then converted to DA by DOPA decarboxylase (**Figure 5A**). After release, DA is taken up into the terminal where it can be degraded by monoamine oxidase (MAO) to produce DOPAC, which can be further catalyzed by catechol-*O*-methyltransferase (COMT) into HVA [**Figure 5A**; also reviewed by (49)]. Interestingly, we found that L-DOPA levels were significantly decreased in EtOH-exposed animals, suggesting that EtOH exposure decreases DA synthesis. This is consistent with a study showing that young adult rhesus monkeys exposed to EtOH in mid-to-late gestation exhibited suppressed DA synthesis (50). Reduced DA synthesis could occur to compensate for repeated EtOH-induced increases in DA release during EtOH exposure (51).

Despite the decreased levels of L-DOPA, DA levels were surprisingly unaltered in the BLA of EtOH-exposed offspring. However, levels of DOPAC and HVA were significantly reduced in EtOH-exposed animals, suggesting that MAO and COMT activity is also reduced, perhaps to compensate for reduced DA synthesis. Studies have reported similar decreases in levels of HVA in the cerebrospinal fluid of rhesus monkeys prenatally exposed to EtOH (52) and in the striatum and frontal cortex of rats exposed to EtOH *in utero* (53). Furthermore, a recent study found that exposure

to a high dose of EtOH during the second trimester-equivalent reduced MAO activity and HVA levels in fetal whole brain, with no changes in DA levels (54). It is also possible that this represents a compensatory mechanism in response to reduced D1R and D3R function, or that reduced DA receptor function could follow changes in DA transmission. Importantly, these findings demonstrate another homeostatic change in the BLA aimed at restoring homeostasis in the DA system following EtOH exposure during the third trimester-equivalent. One caveat about these findings is that these analyte levels were crude measures of tissue content. It would be interesting to further characterize these neurochemical changes using microdialysis and fast scan cyclic voltammetry.

MODERATE POSTNATAL ETHANOL EXPOSURE DID NOT AFFECT ANXIETY-LIKE BEHAVIOR

EtOH-exposed animals did not exhibit alterations in anxiety-like behavior compared to Air-exposed animals on the EPM. Similar findings were shown on an open field in rats exposed to EtOH from P1-P7 via intra-gastric gavage (55). Conversely, exposure to EtOH during the first and second trimesters has been shown to increase anxiety-like behavior (56–59), particularly in response to stress (60). This is generally consistent with human studies as exposure time-dependent effects on anxiety-like behaviors have been suggested to occur in children up to 8 years old that were prenatally exposed to EtOH. Specifically, moderate-to-high EtOH exposure in the first trimester leads to increased odds of anxiety while heavy exposure to EtOH late in pregnancy reduces the odds of anxiety in children prenatally exposed to EtOH (16). Although the molecular or physiological alterations underlying these differences are not clear, exposure during early gestation appears to decrease basal GABA transmission in the BLA (57) and increases dendritic spines on apical dendrites of pyramidal neurons within the BLA (59). In contrast, our study indicates that basal GABA transmission in the BLA is not altered following exposure during the third trimester-equivalent.

DA levels rise in the BLA in response to stressful and/or novel stimuli (5, 6), and DA antagonists alter anxiety-like behaviors. Physiologically, D1R activation enhances inhibition in pyramidal neurons [current study and (11, 26)] presumably to suppress over-excitability of the BLA and behavioral arousal. Consistent with this, a number of behavioral studies have demonstrated that microinjection of D1R antagonists into the BLA results in anxiogenic responses (13). In contrast, D3R activation can disinhibit pyramidal neurons [current findings and (12)], thereby increasing BLA excitability and behavioral arousal. This is supported by behavioral studies that have demonstrated that microinjection of D3R antagonists into the BLA reduce anxiety-like behaviors (12) and taste-related associative learning (61). Furthermore, D3R knockout mice exhibit anxiolytic behavior (14). Taken together, it is clear that D1Rs and D3Rs in the BLA play opposing roles in modulation of anxiety-like behaviors. However, given the compensatory mechanisms in both DA receptor function and DA neurochemistry following EtOH exposure, it is not surprising that EtOH-exposed animals did not exhibit alterations in anxiety-like behavior. It is possible that exposure to higher levels of EtOH could overcome these compensatory mechanisms. Future studies are necessary to assess this possibility.

CONCLUSION

It has been hypothesized that fetal exposure to EtOH leads to hypofunction of the DA system (30, 62–65). DA hypofunction has been shown across species and with exposure occurring at different gestational periods (30), suggesting that this neurotransmitter system is particularly sensitive to EtOH in some brain regions. However, our study suggests that compensatory mechanisms are engaged in the DA system within the BLA to restore anxiety-like responses following exposure to moderate EtOH during the equivalent of the human third trimester, a period when pregnant women often consume EtOH (15). Interestingly, there are other reports of homeostatic changes following developmental EtOH exposure in the cerebellum (66), hippocampus (67–69), and medial septum (70). Future studies should investigate whether under certain conditions (e.g., binge-like heavy EtOH exposure) compensation may fail, contributing to behavioral deficits (71).

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Bovine brain: an *in vitro* translational model in developmental neuroscience and neurodegenerative research

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Animal models provide convenient and clinically relevant tools in the research on neurodegenerative diseases. Studies on developmental disorders extensively rely on the use of laboratory rodents. The present mini-review proposes an alternative translational model based on the use of fetal bovine brain tissue. The bovine (*Bos taurus)* possesses a large and highly gyrencephalic brain and the long gestation period (41 weeks) is comparable to human pregnancy (38–40 weeks). Primary cultures obtained from fetal bovine brain constitute a validated *in vitro* model that allows examinations of neurons and/or glial cells under controlled and reproducible conditions. Physiological processes can be also studied on cultured bovine neural cells incubated with specific substrates or by electrically coupled electrolyte-oxide-semiconductor capacitors that permit direct recording from neuronal cells. Bovine neural cells and specific *in vitro* cell culture could be an alternative in comparative neuroscience and in neurodegenerative research, useful for studying development of normal and altered circuitry in a long gestation mammalian species. Use of bovine tissues would promote a substantial reduction in the use of laboratory animals.

Keywords: brain, neurodegenerative research, translational model, fetal alcohol syndrome, *in vitro* model

ANIMAL MODELS IN RESEARCH ON HUMAN NEURODEGENERATIVE DISORDERS: A BRIEF OVERVIEW

Neurodegenerative diseases are a heterogeneous group of disorders characterized by impairment of neuronal structure and function, and are generally accompanied by neuronal loss.

There is a growing interest in the development of novel animal models (1) and transgenic systems (2), to understand the cellular and molecular basis of human neurodegenerative disorders. Translational medicine is constantly evolving and significant progress has been recently made through the improvement of well-established models and the development of original paradigms (3).

In **Table 1**, we report a selection of reviews focusing on model organisms used in experimental research on human neurodegenerative disorders. Rodents (mice and rats) remain the most widely used species for modeling human neurodegenerative syndromes (4–6). Additional species (cats, dogs, and primates) are used in Parkinson's (7) and Huntington's disease studies (8). Pigs, sheep, and primates are employed besides rodents to study the fetal alcohol syndrome (9).

While rodents are strategic models because of their ease of management, fast reproduction, and low maintenance cost; larger mammals may also be useful because their more complex anatomy and physiology make them more directly comparable to humans in some respects (10). New translational models are also relevant to understand the response to treatment of specific neurodegenerative processes, and essential to better comprehend the natural history of a given disease (1). The goal of this mini-review is to summarize a few issues on the use of the bovine as an alternative experimental model in neurodegenerative research, including the fetal alcohol spectrum disorders (FASDs).

BOVINE: A LARGE MAMMAL WITH A LARGE BRAIN

The bovine species *Bos taurus* is a widespread domestic mammal, raised worldwide for meat and milk production. The bovine possesses a relatively large (approximately 600 g), highly gyrencephalic brain, in comparison to the smooth-surfaced brain of laboratory rodents (11). Furthermore, the CNS of bovine is easily available in large quantities at the slaughterhouse wherever this species is present.

The long gestation period of the bovine (41 weeks) is comparable to human pregnancy (38–40 weeks). During the last decade, our laboratory used this species to study the role of sexual steroids in the regulation of brain differentiation and the expression of cytochrome P450 aromatase, the key enzyme of estrogen biosynthesis (12), in relation to specific estrogen receptor subtypes (ERs). We quantified the expression profiles and neural localization of aromatase P450 and estrogen receptors α and β during consecutive developmental stages in fetal bovine hypothalamus and cerebral cortex (13, 14). Quantitative data analysis on expression patterns of both ERs in different bovine fetal brain regions indicates a strong reciprocal correlation during pregnancy and an increase in the last stage of gestation (14). Our data highlighted that the early second quarter of the gestation (fourth month) is the critical period for hypothalamic differentiation in bovine ontogenesis. This is an important difference with respect to short gestation species

Table 1 | Animal models of human neurodegenerative disorders.

Disorder	Species	Review articles
Alzheimer's disease	Rodents	Gotz and Ittner (5)
Parkinson's disease	Rodents, cats, dogs, and primates	Betarbet et al. (7)
Depression	Rodents	Yan et al. (6)
Schizophrenia	Rodents	Mouri et al. (4)
Huntington's disease	Primates	Aron Badin and Hantraye (8)
Fetal alcohol syndrome	Rodents, pig, sheep, and primates	Cudd (9)

(rat and mouse), where aromatase activity peaks around delivery. In fact, in long gestation species like the bovine and human, the critical period for sexual differentiation occurs in earlier gestation phases (13, 15, 16). It must be noted that the bovine CNS matures comparatively early during pregnancy, since newborn calves must be immediately able to stand, move, and relate to the external world. In contrast, in short gestation species brain differentiation and development continue throughout the perinatal period and afterwards (13, 15, 17). This latter feature only apparently resembles human neoteny, as maturation of the CNS in our species continues for decades but the general organization of the brain is well-established by mid-gestation. The use of fetal bovine brain tissues in experimental medicine may become a valid alternative to laboratory mammals in all those instances in which rodent physiology widely differs from human physiology (see **Table 2**).

An important contribution in remodeling and reshaping of fetal CNS during neural differentiation is performed by the voltage operated calcium channels (VOCCs) that influence cell migration, neuronal sprouting, synaptogenesis, and neurotransmitter release (22–25). The VOCCs are crucial for brain function, and their incorrect expression and/or dysfunction gives rise to a variety of neurological disorders, including pathological pain, epilepsy, migraine, and ataxia (26).

The VOCCs are involved in the maintenance of intracellular Ca^{2+} homeostasis. An increase in intracellular Ca^{2+} triggers a wide range of intracellular processes, such as activation of calcium-dependent enzymes, gene transcription, and neurotransmitter release (27, 28). The properties of the VOCCs are largely conferred by their pore-forming α1-subunits. An impairment of calcium signals is also observed in experimental models of FASD (29). The bovine fetal hypothalamus is a potential tool to evaluate the contribution that VOCCs make to brain development. A recent article assessed the expression of a P/Q and L-type VOCCs by real time RT-PCR, and quantified α1A and α1D subunit expression in the bovine hypothalamus, at various stages of development (30). Data showed that the profile expression of these subunits peaks during the last period of the gestation in the male hypothalamus, in which the expression of α1A and α1D shows higher values than in females. In females, the expression profiles of both genes were constant throughout development (30).

The high expression of α1A and α1D during development suggests that the presence of an increased density of P/Q and L-type VOCCs, which may be involved in the process of sexual differentiation during development, an hypothesis also supported by other studies (22). Sex differences in the levels of L and P/Q channel expression may be a part of the mechanism leading to the onset of activities that control differentiation in young CNS neurons. Moreover, their activity may be crucial for physiological responses of neuronal populations, starting from the second half of the pregnancy when the architecture of bovine hypothalamus is defined and networks start to develop.

Table 2 | Use of bovine tissues for the study of altered sexual differentiation and neurodegenerative research.

Neurodegenerative disorder	Review
Twin–twin transfusion syndrome	Padula (18)
Batten disease	Weber and Pearce (19)
Neuronal ceroid lipofuscinosis	Bond et al. (20)
Prion diseases	Imran and Mahmood (21)

BOVINE BRAIN: A TRANSLATIONAL MODEL FOR ALTERED SEXUAL DIFFERENTIATION OF THE BRAIN AND NEURODEGENERATIVE RESEARCH

Large animals are more similar to humans in relation to brain size and lifespan and could be therefore essential to investigate complex patho-physiological mechanisms relating to neurodegenerative diseases and infectious neuropathologies (31).

The bovine freemartin syndrome, the most frequent form of intersexuality found in cattle, may represent a useful model in which to study the human condition called twin–twin transfusion syndrome. Freemartins develop when vascular connections are established between the placentas of developing heterosexual twin fetuses, and the result is masculinization of the female reproductive tract to varying degrees due to the high circulating levels of testosterone (18). These natural born intersex calves could represent an ideal model to study sexual patho-physiological evolution of sexual brain differentiation in mammals. These bovine pseudohermaphrodite females are a common instance in bovine twin pregnancies (involving one male and one female fetus) and can also be artificially induced.

In humans, twins originating from a single placenta form vascular anastomoses, which may lead to unequal sharing of blood supply and ultimately the impaired development or death of one or both fetuses (32). The infrequent nature of the condition makes comparison of treatment options difficult and the bovine freemartin may represent an animal system in which to study and compare treatments. It is worth noting here that recent findings suggest the possibility that early alcohol exposure may have steroid-mediated sexually dimorphic effects on serotoninergic neurons (33).

A recent review (34) examined the use of non-laboratory or large animal models for neuronal ceroid lipofuscinoses (NCL; Batten disease), a group of fatal progressive neurodegenerative diseases predominantly affecting children. Data from the literature confirm that natural cases of NCL occur in a large variety of species including the bovine (19, 20). Research in prion pathology, the transmissible neurodegenerative conditions affecting human and a wide range of animal species, lead to an increased awareness

of the need to use large animal models such as the bovine, in addition to conventional laboratory animals (21).

IN VITRO TOOL FOR NEURODEGENERATIVE STUDIES

In vitro models provide important insights into the pathogenesis of neurodegenerative disorders and represent an interesting approach for the screening of potential pharmacological agents (35, 36). To obtain scientifically valid research, experimental conditions must be strictly controlled: this often involves manipulating one single variable at a time while keeping the others constant, and then observing the consequences of that single specific change. To this effect, primary cultures from fetal bovine hypothalamus and cerebral cortex may be standardized to obtain a reliable and reproducible model.

A number of studies have validated *in vitro* models based on neural primary cultures obtained from fetal bovine hypothalamus, cerebral cortex, and cerebellum, allowing examinations of neurons and/or glial cells under controlled and reproducible conditions. Cell cultures obtained from frozen–thawed bovine fetal tissues are comparable to cultures derived from fresh fragments of cortex and hypothalamus of the same animal, showing similar growth profiles (37, 38). Bovine cultures from the hypothalamus and frontal cortex retain also *in vitro* the ability to express and synthesize the enzyme aromataseP450Arom and the α- and β-estrogen receptors (17). These data are in agreement with data observed in others species, such as mice, rats, and avian species (39–41). Bovine neurons *in vitro* maintain the ability to generate action potentials. Electrolyte-oxide-semiconductor capacitors (EOSCs), a class of microtransducers for extracellular electrical stimulation, may be employed to activate voltage-dependent sodium channels at the neuronal soma, resulting in a versatile complement for the investigation of Ca^{2+} signaling (42).

Bovine cerebellum-derived endothelial cell lines are useful to monitor Ca^{2+} oscillations in the main intracellular compartments including the cytosol, the endoplasmic reticulum, and the mitochondria. Mitochondrial Ca^{2+} uptake significantly decreased after 48-h exposure to estradiol, whereas cytosolic and endoplasmic reticulum responses were unaffected. The permeability transition pore (PTP) may be involved in the mechanism of action and influences energy metabolism and cell viability. Treating cells with cyclosporine A (CsA), which binds to the matrix chaperone cyclophilin-D and regulates PTP opening, reversed the effects of a 48-h treatment with estradiol, thus suggesting a possible transcriptional modulation of proteins involved in the mitochondrial permeability transition process (43).

Fetal alcohol spectrum disorder has also been considered a neurodegenerative disease [see Ref. (44)] with an interesting mechanism involving glutamate receptors and excessive activation of GABA(A) receptors and consequent apoptotic neurodegeneration in the developing rat forebrain. This process could be replicated in bovine primary cultures by defining the proper synaptogenetic phase. Since vulnerability to ethanol exposure coincides with the period of synaptogenesis, which in humans starts from the sixth month of gestation (44), bovine fetal tissues could represent a standardized model and a dynamic system to study molecular mechanisms and physiological process at the cellular level, and potentially practical also for drug discovery.

IMPLICATIONS FOR ANIMAL WELFARE

The use of experimental animals in biomedical research follows precise national regulations that are increasingly based on the three "Rs" principle (replacement, reduction, and refinement). Investigations on neurodegenerative disorders extensively use laboratory rodents, and reluctance to consider alternative species may derive from a cultural bias.

Our review proposes the use of fetal and adult bovine brain tissue as a potential alternative translational model. Bovine neural tissues employed for experimental studies have the further advantage to be easily obtained in large quantities from slaughterhouses, allowing a considerable reduction in the sacrifice of laboratory animals. Fetal tissues are also widely available, due to the frequent accidental slaughtering of undiagnosed pregnant cows.

A fundamental goal of the Animal Welfare Act is the minimization of animal pain and distress by use of alternative methods. We considered this ethical point of view as the initial criteria of the present mini-review, promoting the development and validation of this new and alternative translational model. In this sense, the use of brain slices is a recognized tool in neurodegenerative investigations (45, 46).

CONCLUSION

In this view, the bovine neural cells and specifically the *in vitro* cell cultures could be an alternative of interest in developmental neuroscience and consequently a potential tool for studying the pathophysiology of altered circuitry linked to fetal alcohol exposure during pregnancy (47) in a dynamic system and under standard conditions. Bovine tissues may represent also a novel resource for the study of neurodegenerative disorders.

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Focus on apoptosis to decipher how alcohol and many other drugs disrupt brain development

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Maternal ingestion of alcohol during pregnancy can cause a disability syndrome termed fetal alcohol spectrum disorder (FASD), which may include craniofacial malformations, gross structural brain pathology, and a variety of long-term neuropsychiatric disturbances, or it may consist of subtle brain changes and neuropsychiatric disturbances in the relative absence of gross dysmorphogenic features. Based on a large body of recent evidence, we have proposed (1) that most, if not all, of the deleterious effects of alcohol on the developing brain can be explained by a single mechanism. Alcohol has apoptogenic properties that cause large numbers of CNS progenitor cells, or fully differentiated brain cells (depending on developmental age at time of alcohol exposure) to commit suicide and be deleted from the pool of cells that would ordinarily survive and contribute to the normal functions of the brain. If excessive cell suicide is triggered by alcohol in a very early stage of development, the result, as Sulik and colleagues have shown (2), will be gross dysmorphogenic anomalies (e.g., craniofacial and midline brain anomalies), because the cells deleted are progenitor cells that are responsible for generating cell populations that comprise the building blocks of these craniofacial and brain structures. But if, as we have demonstrated (3–5), alcohol triggers suicide of CNS cells in later stages of development after these cells are already differentiating into neurons and glia, the result will be a reduced number of brain cells, derangement of brain circuitry, and various neuropsychiatric disturbances, depending on which populations of cells have been deleted and what combination of synaptic connections have been disrupted or destroyed.

Alcohol's apoptogenic action is linked to its NMDA glutamate antagonist and $GABA_A$ agonist properties. Many other drugs that have one or both of these properties also trigger developmental apoptosis, including other drugs of abuse (phencyclidine, ketamine, benzodiazepines, and barbiturates), and many drugs used in obstetric and pediatric medicine [all sedative/anesthetic drugs (SADs), and most anti-epileptic drugs (AEDs)] (3, 6–8). It was demonstrated quantitatively in early studies that neurons are permanently deleted from the developing brain by exposure to these drugs, and that brain volume is permanently reduced and synaptic ultrastructure disrupted. No region of the central nervous system is totally spared, in that the degenerative response has been demonstrated in neurons distributed widely throughout the forebrain, midbrain, cerebellum, brainstem, spinal cord, and retina (3, 4, 9–11). Although alcohol's apoptogenic action was originally thought to impinge only on neurons, it is now well established that oligodendrocytes (oligos), distributed diffusely throughout the white matter, also succumb to apoptosis following developmental exposure to alcohol or to SADs or AEDs (12–16). The injury induced by apoptogenic drugs is dose and developmental age-dependent, with several different patterns of neuronal degeneration observed, depending on developmental age at time of drug exposure. The cell death process involves Bax-mediated extramitochondrial leakage of cytochrome *c* (17), which is followed by a sequence of changes culminating in the activation of caspase-3 (5, 18). An important property that apoptogenic drugs have in common is that they rapidly suppress phosphorylation of extracellular signal-regulated kinases (ERK) (signaling system that regulates cell survival) in the *in vivo* developing brain. This has potentially important implications for preventing this type of brain injury, in that lithium counteracts the suppressant action of apoptogenic drugs on pERK (19), and also protects against apoptogenic injury induced by these drugs in the infant mouse (19–21) or infant monkey brain (22).

The developing rhesus macaque brain is quite sensitive to the toxic action of apoptogenic drugs, and in both rodents and monkeys two specific cell types are affected – neurons and oligos – and the mode of cell death for both cell types is apoptosis. Many of the structural brain changes reported in children with FASD are also seen in the brains of rodents and monkeys following exposure to alcohol and related apoptogenic drugs [illustrated extensively in Ref. (1)]. A prime example of a prominent structural brain change caused by alcohol and all other apoptogenic drugs following exposure of the primate brain in the early third trimester is loss of basal ganglia (BG) neuronal mass. This has long been recognized as a prominent finding in children who were exposed *in utero* to alcohol (23, 24), and also has been reported in children who were exposed to AEDs in the third trimester of gestation (25), and in premature infants who have learning disability following exposure to surgical anesthesia (26) or following prolonged sedation in the neonatal intensive care unit (27).

The window of vulnerability in primates appears to be very similar for all of these drugs. Valproate, an AED with very strong apoptogenic properties (7), mimics alcohol in causing craniofacial and midline brain anomalies following human exposure in

the first trimester (28), and in causing a large IQ deficit following human exposure in the third trimester (29, 30). SADs have not been studied adequately for early dysmorphic effects, but we have shown that alcohol and numerous SADs (isoflurane, propofol, ketamine, benzodiazepines, and barbiturates) trigger a robust apoptosis response in the fetal monkey brain on gestational days 100–120 (comparable to human late second trimester), and vulnerability continues throughout the third trimester and up to a yet to be established age after birth (12–16, 31, 32). Mounting evidence from animal studies prompted a series of recent human studies, which have documented that brief anesthesia exposure of premature infants (26), or full term human infants (33–40) is associated with increased risk for neurocognitive deficits. Thus, it is clear that apoptogenicity is a property that alcohol and certain other drugs have in common, and emerging evidence suggests that in both early and late gestation these drugs have the potential to cause FASD-like structural brain changes and FASD-like neurodevelopmental disability syndromes. Available evidence suggests that FASD syndromes induced by anesthetic drugs are usually less severe than the syndrome that alcohol often causes, the obvious reason being that pregnant mothers who have a strong alcohol habit expose their fetuses multiple times during gestation to prolonged "binge" blood levels of alcohol, whereas the vast majority of human infants or fetuses who are exposed to anesthetic drugs are exposed only once for a relatively brief duration. Consistent with this thesis, the numerous human studies cited above are in good agreement that risk for poor neurocognitive outcome is greater following multiple anesthesia exposures than following a single exposure.

Although many mechanisms have been proposed to explain the FASD syndrome, the only mechanism identified, thus far, that can actually explain most if not all of the brain and behavioral pathology comprising that syndrome can be summed up in a single word – apoptosis. Within only a few hours after alcohol enters the developing brain, millions of brain cells that were on a healthy survival track, suddenly become derailed and commit suicide. The cells that die belong to both the neuronal and oligo lineages. Oligos are vitally important for normal neuronal function. Although widespread loss of neurons, or their progenitors, from the developing brain would be a sufficient mechanism to explain the signs and symptoms of FASD, simultaneous deletion of oligos, or their progenitors, makes the case even stronger for apoptosis as a single primary mechanism that can explain all features of the FASD syndrome. Once the apoptotic deletion of neurons and oligos (or their precursors) has occurred as the primary injurious event, there are numerous secondary mechanisms that come into play as the brain attempts to compensate for the disruptive influence of this primary injury. For example, loss of neurons causes an impoverishment of dendritic fields for receiving synaptic inputs from incoming axons, and loss of neurons also means there will be fewer axons to establish those synaptic contacts (41, 42). Developing brain networks must reconstitute and reorganize themselves to cope with this primary insult. Researchers can spend lifetimes studying the myriad steps in this reorganization process, but identifying these many features of the deranged and reorganized circuitry, will not yield insights necessary for preventing alcohol (or SADs and AEDs) from causing the initial injury and consequent derangements. The scenario I have just described pertains to a single episode of alcohol exposure. Consider how complicated the reorganization task will be for the brain of a fetus whose mother heavily abuses alcohol multiple times, both early and late, during pregnancy. Again, much time can be spent in studying this multi-layered complex reorganization process, but if the end goal is to learn how to prevent this type of developmental injury, the time will be better spent focusing on apoptosis as the primary cause, and deciphering the molecular mechanisms by which alcohol (or SADs and AEDs) unleash the apoptosis cascade. A better understanding of these mechanisms can lead to effective methods for preventing apoptogenic drugs from injuring the developing brain.

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Alterations of neocortical pyramidal neurons: turning points in the genesis of mental retardation

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Pyramidal neurons (PNs) represent the majority of neocortical cells and their involvement in cognitive functions is decisive. Therefore, they are the most obvious target of developmental disorders characterized by mental retardation. Genetic and non-genetic forms of intellectual disability share a few basic pathogenetic signatures that result in the anomalous function of PNs. Here, we review the key mechanisms impairing these neurons and their participation in the cortical network, with special focus on experimental models of fetal exposure to alcohol. Due to the heterogeneity of PNs, some alterations affect selectively a given cell population, which may also differ depending on the considered pathology. These specific features open new possibilities for the interpretation of cognitive defects observed in mental retardation syndromes, as well as for novel therapeutic interventions.

Keywords: apoptosis, dendrites, calcium spikes, fetal alcohol spectrum disorders, dendritic spines

Santiago Ramón y Cajal referred to the neocortical pyramidal neuron (PN) as "La noble y enigmática célula del pensamiento" (the noble and enigmatic cell of thought) (1). These glutamatergic, excitatory neurons represent the vast majority of neocortical cells (about 80–90%), the remaining being constituted by GABAergic, inhibitory interneurons. Surprisingly and contrary to what one may expect, cortical interneurons, though minor in number, are characterized by a great variety of anatomical features, electrophysiological properties, and synaptic attributes [see Ref. (2) for review]. Conversely, PNs are often conceived as a rather homogeneous population. However, the principal neurons of the cerebral cortex are far from being identical to each other, since they show both evident and more subtle differences (**Figure 1**). In the present mini-review, we will first provide some examples of how PNs represent a heterogeneous population. Then, while it is quite obvious that developmental disorders associated with mental retardation (MR) target the main structure involved in cognitive functions (i.e., the cerebral cortex) and its majority neurons, we try to answer the question whether given subpopulations or functional features of PNs are preferentially affected. We focus mainly on the effects of fetal exposure to alcohol (see **Figure 2**), highlighting analogies and differences with other developmental disorders associated with MR.

HETEROGENEITY OF PNs

The difference among PNs is already apparent at a first glance of histological sections and is related to their radial position within the six-layered neocortical sheet. Besides the obvious morphological difference (short vs long apical dendrites), supragranular (layer 2/3; L2/3) and infragranular (layer 5; L5) PNs participate differently to the flow of information in the canonical microcircuit of the cortical column (8). Differences between supra- and infragranular layers can be observed also when looking at the more subtle, intrinsic electrophysiological properties. For instance, L2/3 neurons display less hyperpolarization-activated currents (I_h), compared to L5 neurons (9).

The analysis of the fine columnar connections makes it possible to further distinguish subpopulations within L2/3 neurons. In the barrel cortex, for instance, lemniscal and paralemniscal afferents target PNs located at different depths in the supragranular layers (10). In the rodent visual cortex, L2/3 neurons are selectively interconnected to form fine-scale, distinct subnetworks (11).

Layer 5 PNs can be also further subdivided into subsets featuring discrete properties. Based on morphology, electrophysiology, and functional connectivity, L5 PNs are classified into intrinsically bursting and regular spiking. The former have the tendency of firing bursts of action potentials in response to steps of depolarizing current, usually display a prominent apical tuft in layer 1 (thick-tufted), and project to subcortical targets. The latter fire trains of action potentials with constant interspike intervals, have a slender apical dendrite, and project mainly to other cortical areas (12–14). Within layer 5, PNs belonging to the sparse L5a and the densely populated L5b are also clearly distinguishable, according to differences concerning functional and connectional properties (15, 16). Even when L5 PNs project to the same subcortical target, they may be involved in different functional circuits, as it has been proposed for cortico-striatal neurons sustaining the direct and indirect pathways of the basal ganglia, respectively [(17); but see Ref. (18)]. The parcelation of PNs according to their radial distribution is further complicated by the heterogeneous population of layer 6 neurons (19). The apical dendrites of these cells are unusual, as they, unlike those of other PNs, do not reach superficial layers, although sharing many electrophysiological properties with other neocortical PNs (20).

If the uneven properties of PNs along the radial cortical dimension reflect the structure-function relationship within the column microcircuit, equally outstanding is the diversity along the tangential dimension. In this regard, the complexity of the dendritic tree

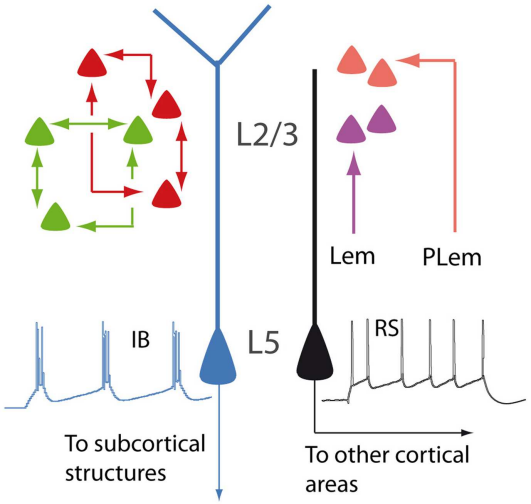

FIGURE 1 | Schematic illustration showing different types of PNs. In layer 2/3, neurons are interconnected to form distinct subnetworks (green and red cells). In the barrel cortex, lemniscal (Lem) and paralemniscal (PLem) afferents target different subpopulations (purple and orange cells). In layer 5, regular spiking PNs (RS, black) and intrinsically bursting PNs (IB, blue) display different dendritic morphologies and different projections.

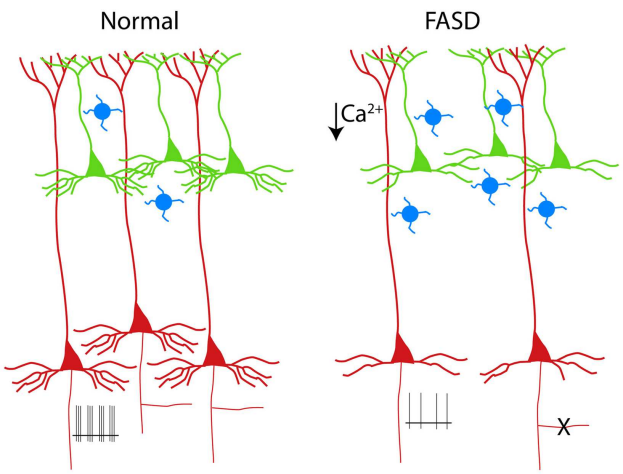

FIGURE 2 | Schematic drawing of PNs in a normal cortical column (left). The alterations of PNs observed in experimental models of FASD are shown on the right (3–6). Supragranular and infragranular PNs are shown in green and red, respectively. Note the reduced population of L5 PNs (or the pruning of their axon collateral, **X**) and the simplification of the basal (but not apical) dendrites. The hypoexcitability of L5 PNs (shown as reduced number of spikes at the axon level) is a consequence of reduced dendritic calcium spikes (5). The increased number of calretinin interneurons (blue cells) is also shown (7).

increases as one moves from primary sensory to higher order areas, reaching the most complex pattern in the prefrontal cortex (21). Further, the prefrontal cortex contains a large number of unusual PNs, which display an early bifurcation of the apical dendrite, whose total length is therefore susbstantially increased (22).

We have briefly outlined the laminar and regional heterogeneity of PNs. However, the reader should bear in mind that, even if neocortical PNs were homogeneous across cortical areas and layers, nonetheless each of them would represent the most complex neuron of the mammalian brain. Let us consider, for example, the L5 PN. Its apical dendrite extends through most of cortical thickness and is thus ideally suited for translaminar integration. In addition, the long, apparently homogeneous dendritic arbor of these neurons features specific functional properties: basal dendrites and the apical tufts are dominated by NMDA spikes, while Ca^{2+} spikes sustained by voltage-gated channels prevail in the distal apical trunk (23). Finally, dendritic, axon, and somatic domains of L5 PNs are targeted by different types of inhibitory interneurons (24). In summary, even the single PN is a complex world itself, able to integrate feedforward ascending input and feedback connections to generate the cognitive performance (25).

APOPTOSIS

Early exposure to alcohol, whose effects are globally referred to as fetal alcohol spectrum disorders (FASD), are well known causes of mental retardation. There are manifold factors involved in the neurodevelopmental toxicity of ethanol, which is critically dependent on the dose and time of exposure [see Ref. (26), for review]. Experimental models of FASD allow a tight control of alcohol exposure and help to dissect out the mechanisms operant at different developmental stages. When rodents are exposed during prenatal life, alcohol is more likely to interfer with the proliferation of neuron precursors and/or with the migration of cortical cells (27, 28). By contrast, when rodents are given alcohol during the first two postnatal weeks [corresponding to the third trimester of gestation in humans, see Ref. (29)], a massive apoptosis occurs in several brain structures, including the cerebral cortex (30). The third trimester equivalent is characterized by intense synaptogenesis and the alcohol-induced apoptosis is thought to be caused by the simultaneous blockade of NMDA receptors and activation of GABA receptors (31). The apoptosis observed in the neocortex after postnatal alcohol exposure in rodents seems to affect mainly infragranular PNs, as demonstrated by the selective presence of molecular markers of apoptotic susceptibility, such as caspase 3 and the low-affinity neurotrophin receptor (p75 NTR), in L5 cells [(3, 32); see **Figure 2**]. The prevailing involvement of infragranular PNs is also suggested by the increased ratio between supragranular and infragranular PNs sustaining the cortico-cortical associative projections (4). Notably, the vulnerability of these neurons to apoptosis outlasts the alcohol exposure, since an increased immunoreactivity for p75 NTR is observed several days after withdrawal (3). In a different experimental model of MR, reproducing the congenital hypothyroidism, the increased apoptosis is associated to upregulation of p75 NTR (33). In this case, however, the apoptotic cells are confined to supragranular instead of infragranular layers (33).

The unbalanced weights of supra- and infragranular layers, as observed in different types of MR, can yield important functional consequences. For instance, sensory and memory processing carried out by the same cortical area are mediated by opposite flows of interlaminar signals [supragranular → infragranular and infragranular → supragranular, respectively; see Ref. (34)].

It is worth noting here that experimental models mimicking other types of MR are characterized by a reduced rate of naturally occurring cell death, rather than by increased apoptosis. This is the case for FMR1 mutants (reproducing the fragile X syndrome) and for the Rett syndrome as well (35, 36). Thus, it appears that both the excess of apoptosis and the lack of programed cell death can equally lead to an impairment of the cortical network and to cognitive defects.

DENDRITES AND CONNECTIVITY

The dendritic tree of PNs, with its long and extensively ramified branches, must be considered the main computational device of the neocortex (37). Therefore, it is not surprising that dendritic alterations are recognized as the key anatomical counterpart of MR (38). In experimental models of FASD based on early postnatal exposure, the basal dendritic arbor of PNs is more affected, as compared to the apical dendrite [(4, 5); see **Figure 2**]. Basal dendrites of L2/3 associative PNs in alcohol-treated rats display fewer dendritic branches than in controls, suggesting a defect of branching rather than of terminal dendrite elongation (6). This dissociation can be justified by the different molecular machinery involved in the two distinct phenomena of branching and terminal elongation (39). In the Ts65Dn mouse model of Down syndrome, the basal dendrites of L2/3 PNs, similarly to what observed in FASD, display a reduced complexity of the branching pattern (40). However, in humans affected by Down syndrome, dendritic alterations follow a complex temporal sequence, resulting in a simplification that is more dramatic for apical dendrites (41). A Golgi study by Armstrong and coworkers (42) provides a direct comparison between the dendritic anomalies of Rett and Down syndrome, pointing out that basal dendrites of the frontal cortex in individuals affected by Rett syndrome are strongly impaired both in supra- and infragranular layers, while apical dendrites are affected only in supragranular layers. In experimental models of early-onset hypothyroidism, finally, both apical and basal dendrites of PNs appear to be strongly reduced (43).

Understanding which dendritic domain of PNs is preferentially targeted by disorders associated to MR is not trivial. In fact, basal and apical dendrites not only display different branching patterns, but are also characterized by different functional properties and are likely to play distinctive roles in the cortical network. Apical dendrites receive long-range feedback input from higher order cortical areas (44) and display both Ca^{2+} and NMDA spikes, whereas basal dendrites support only NMDA spikes (45).

Another central issue concerning the relationship between dendrites and MR is represented by the density and distribution of dendritic spines. Most inputs synapsing upon PNs occur on these small protrusions, which are essential for the linear summation of excitatory potentials (46). Almost all disorders associated with MR feature alterations of the number and/or shape of dendritic spines (38). Although a systematic review of dendritic spine anomalies is beyond the aim of the present paper, it is worth mentioning that both a decreased and an increased number of spines can lead to MR. While a reduction of dendritic spines has been observed in experimental models of FASD [e.g., Ref. (47)], their number is significantly higher in fragile X mice (48). Once again, as already pointed out for neuronal populations (see above), also the dendritic spines seem to ensure the good functioning of PNs only if they reach an optimal number. Fewer or more spines, conversely, can equally lead to defective function.

Since each spine is thought to represent the site of at least one synaptic contact, quantitative and/or qualitative spine anomalies are likely to reflect alterations of cortical connectivity. Thus, dendritic alterations can be accompanied by a defect of axon outgrowth or pruning, as demonstrated for early exposure to ethanol (49, 50), for mouse models of Rett syndrome (51), and fragile X syndrome (52). The obvious consequence is a modified intracolumnar (53) and long-range connectivity (4). The main alterations observed in experimental models of MR are summarized in Table S1 in Supplementary Material.

PN EXCITABILITY

The excitability of PNs (i.e., the ability of generating action potentials in response to depolarizing current) depends primarily on the intrinsic membrane properties and, to some extent, on the cited complexity of the dendritic tree. In fact, PN dendrites are not merely passive cables, but they are also endowed with a great variety of active conductances (54). Dendritic voltage-gated channels, in turn, can influence the axo-somatic firing pattern of PNs (55). We have demonstrated that exposure to ethanol during the third trimester equivalent leads to a long-lasting reduction of excitability in L5 PNs (5). Such an impairment represents the consequence of decreased spikes in the Ca^{2+} electrogenesis zone of the apical dendrite. These spikes are usually mediated by voltage-gated Ca^{2+} channels and are accompanied by their somatic counterpart, consisting of a prominent afterdepolarization. Interestingly and in agreement with our observation, Sánchez-Alonso et al. (56), in a mouse model of congenital hypothyroidism, noted that hippocampal PNs showed a decreased afterdepolarization.

An alteration of Ca^{2+} signaling has been also observed in experimental models of fragile X syndrome (57). This condition, however, is rather characterized by hyperexcitability (58). Besides affecting the neuron excitability, the unreliability of Ca^{2+} signals can alter the neural plasticity, as consistently observed in experimental models of MR (57, 59, 60).

CONCLUDING REMARKS

It seems pretty clear that the different etiological factors involved in different types of MR converge upon a few basic mechanisms, regardless of the vast variety of molecular pathways leading to such disturbances. Most of these alterations impair the functional properties of the major cell type of the neocortex, i.e., the PN. Here, we have briefly described some of the main mechanisms at the basis of MR, concerning the number, the dendritic tree, the connections, and the excitability of PNs. However, the picture can be complicated by the possibility that some of the described alterations affect selectively discrete populations of PNs, or even discrete subregions of the same cell.

A further contribute to the complexity derives from the obvious consideration that, despite their high number, PNs are not the only determinant of cortical network properties. In fact, the interplay between PNs and GABAergic interneurons is a key element of cortical physiology (24). Early exposure to alcohol results in a change of cortical interneurons, with a significant increase

of calretinin cells (7). These neurons usually co-express VIP and contact other interneurons, thus mediating disinhibition of PNs, possibly driven by feedback input from higher cortical areas (61). Therefore, the decreased intrinsic excitability of the distal apical dendrite observed in FASD (5) can be counterbalanced under certain circumstances by a relative increase of the network-mediated disinhibitory pathway.

Another puzzling issue is the apparently opposite tendency of some anatomical and electrophysiological properties in different forms of MR, as is the case for hypo- and hyperexcitability. However, this is not necessarily a contradiction, at least in terms of the functional outcome. In fact, both hypo- and hyperexcitability can equally contribute to flatten the current-frequency curve, with a reduction of the dynamic range of PNs and a consequent impairment of the ability to encode relevant information (62).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fped.2014.00086/abstract

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Exploring the complexity of intellectual disability in fetal alcohol spectrum disorders

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Brain development in mammals is long lasting. It begins early during embryonic growth and is finalized in early adulthood. This progression represents a delicate choreography of molecular, cellular, and physiological processes initiated and directed by the fetal genotype in close interaction with environment. Not surprisingly, most aberrations in brain functioning including intellectual disability (ID) are attributed to either gene(s), or environment or the interaction of the two. The ensuing complexity has made the assessment of this choreography, ever challenging. A model to assess this complexity has used a mouse model (C57BL/6J or B6) that is subjected to prenatal alcohol exposure. The resulting pups show learning and memory deficits similar to patients with fetal alcohol spectrum disorder (FASD), which is associated with life-long changes in gene expression. Interestingly, this change in gene expression underlies epigenetic processes including DNA methylation and miRNAs. This paradigm is applicable to ethanol exposure at different developmental times (binge at trimesters 1, 2, and 3 as well as continuous preference drinking (70%) of 10% alcohol by B6 females during pregnancy). The exposure leads to life-long changes in neural epigenetic marks, gene expression, and a variety of defects in neurodevelopment and CNS function. We argue that this cascade may be reversed postnatally via drugs, chemicals, and environment including maternal care. Such conclusions are supported by two sets of results. First, antipsychotic drugs that are used to treat ID including psychosis function via changes in DNA methylation, a major epigenetic mark. Second, post-natal environment may improve (with enriched environments) or worsen (with negative and maternal separation stress) the cognitive ability of pups that were prenatally exposed to ethanol as well as their matched controls. In this review, we will discuss operational epigenetic mechanisms involved in the development of intellectual ability/disability in response to alcohol during prenatal or post-natal development. In doing so, we will explore the potential of epigenetic manipulation in the treatment of FASD and related disorders implicated in ID.

Keywords: neurodevelopment, fetal alcohol spectrum disorders, mouse models, epigenetics, stress, environmental enrichment, intellectual disability, gene expression

INTELLECTUAL DISABILITY

Mental retardation is a highly diverse group of cognitive disorders. The fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (*5th ed.*; *DSM-5*) characterizes mental retardation [revised as Intellectual Disability (ID) in the fifth edition) by impairments of general mental abilities that fall under the conceptual, social, and practical domains of adaptive functioning. Individuals with ID have intelligence quotient (IQ) scores below 70, approximately two standard deviations or more below the population average score (1). They represent 1–3% of the population in Canada, and males are affected more often than females (2). Roughly two-thirds of ID individuals have mild-to-moderate impairments while the remaining third are severely affected (2). In 2006, the Participation and Activity Limitation Survey by Statistics Canada, found the largest proportion of ID occurs in the 15–24 age group (29.3%) (2). The survey also found that compared to individuals with physical disabilities (52.7%), people with intellectual disabilities are far less likely to be employed (26.1%) with an inverse trend of socio-economic status with respect to prevalence (2). In America, the lifetime costs are expected to be near $50 billion for individuals born in 2000 with ID (3).

Intellectual disability and its variable manifestations are often attributed to aberrations in neurodevelopment that are complex, poorly understood, and long lasting in mammals. It begins early during embryonic development but may take years to complete and is finalized in early adulthood. Further, it involves a delicate choreography of cellular, molecular, and physiological processes directed by the fetal genotype in close interaction with the environment at every step, over time. Consequently, it covers periods before birth, during birth, and/or the childhood years. The causes of ID are complex and multifactorial. In some rare cases, the primary determinants of ID are known. For example, rare chromosome number defects [Down syndrome (4, 5)], inherited chromosomal disorders [Fragile X syndrome (6)], and a number of single gene mutations (7–9) are known to cause a spectrum of intellectual. Unfortunately, ID tends to be heterogeneous with

a wide spectrum of manifestations. Additionally, neurodevelopmental exposure to a variety of drugs and chemicals can result in ID, such as cocaine, alcohol, and lead, among others (10). ID is not a single disorder, rather the result of a plethora of causations involving both genes and environment. The understanding of the developmental processes associated with ID and related abnormalities calls for a research focus on specific diagnosis potentially caused by a single known factor, an experimental model that is easier to manipulate and interpret. In this discussion, we will use fetal alcohol spectrum disorders (FASD) as a case study of the complexity of ID.

FETAL ALCOHOL SPECTRUM DISORDERS

Fetal alcohol spectrum disorder with all its manifestations results from a single initial cause, prenatal alcohol exposure (PAE). It includes mild behavioral and learning impairments, to the most severe form called Fetal Alcohol Syndrome (FAS). FAS may include ID as well as birth defects (11). The intellectual deficits in FAS and FASD are highly variable and heterogeneous. These symptoms are considered chronic, often co-occurring with other mental impairments, and manifesting during the developmental period. FASD represents one of the most common causes of learning disabilities, cognitive deficits, and ID (12). The severity of impairments is evaluated using both clinical assessments and standardized testing of intelligence.

Obtaining a diagnosis of FASD requires input from various medical professionals, with estimated costs associated with the diagnostic procedure up to $7.3 million per year in Canada (13). Unfortunately, despite increasing public education on the dangers of PAE, the occurrence of alcohol exposed pregnancies remains a significant societal problem. In Canada, 74.4% of women surveys reported alcohol use in the past year (14). Most disappointing is the prevalence of PAE in certain high-risk groups. In Fort McMurray, Alberta, almost 50% of pregnant women surveyed reported consuming an alcoholic beverage since learning of their pregnancy (15). In a survey of women in Arctic Quebec, over 60% of women reported alcohol consumption during pregnancy (16). While the rates of PAE are often considered high, not every reported incident of alcohol exposure results in FASD. In Canada, the more severe FAS is estimated to occur at rates of 1–2 per 1000 live births (17), while the more mild FASD occurs much more often, at a rate of 9 per 1000 (18). In northeastern Manitoba, estimates of FASD incidence are as high as 14.8 per 1000 births (19). Children entering child care systems, such as foster care and orphanages, also represent a subpopulation with higher incidence of FASD, with estimates at 60 per 1000 children (20). A 2010 study found that 48% of pregnancies in the United States were unintended (21), and over 30% of women reported consuming alcohol while pregnant (22). The Centre for Disease Control and Prevention reports FAS rates in the USA ranging from 0.2 to 1.5 per 1000 live births solidifying the position of FAS as one of the leading preventable causes of intellectual disabilities (23).

How neurodevelopmental alcohol exposure may cause ID is poorly understood. It is a critical area of research. Such studies are not always feasible in humans. In this review, we will present arguments to suggest that studies on the mechanisms in the development of intellectual disabilities could be modeled in suitable animal models using PAE. It allows coverage of prenatal as well as post-natal development. Specifically, we will focus on behavioral data to show that B6 mice offer an opportunity to assess the effect of neurodevelopmental time specific PAE on molecular processes that are affected by alcohol and may lead to the manifestation of ID and related abnormalities. Additionally, it allows controlled post-natal manipulation (negative stress or positive enrichment) on the manifestation of mental deficits in pups generated with and without PAE.

MOUSE MODEL OF FASD RESEARCH

It is understandable that most research on the mechanisms involved in the development of FASD has concentrated on animal models, particularly mice (24–27). To this end, our laboratory has established two forms of neurodevelopmental *in vivo* alcohol treatment in B6 mice. The first uses injections at any time during neurodevelopment on time-mated females and the second uses free choice of 10% alcohol or water as the source of liquid for pregnant females. The pregnant B6 mothers prefer (~70%) to drink a 10% ethanol in water solution over water. The two methods equate to the two forms of PAE in humans; binge (injection) at any time during pregnancy and continuous maternal drinking (preference) during pregnancy. The resultant pups from the two treatments show alcohol specific phenotypes; developmental delays, increased anxiety, learning deficits, and pronounced deficits in visuo-spatial memory (27–29). They also exhibit delayed neural reflexes, aberrant limbic coordination, elevated levels of anxiety, and spatial-memory deficits (27). To better ascertain the effects of ethanol on critical neurodevelopmental time points, we have mimicked binge-like drinking episodes at critical times, representing equivalents to the three trimesters in humans. The trimester three equivalent represents a "brain growth spurt" – dominated by synaptogenesis during the first 2 weeks in B6 newborn pups (29, 30). It is a period marked by the formation of extensive neural connections that form the basis for much of the cell-to-cell communication in the brain. The ability of ethanol to trigger widespread neurodegeneration during synaptogenesis is accompanied by the upregulation of stress-related and apoptosis-related genes and a down-regulation of genes related to protein synthesis, mitosis, synaptic formation, and maintenance (28, 30, 31). The third trimester equivalent ethanol exposure also results in increased anxiety-like behavioral traits and pronounced recognition memory and visuo-spatial memory defects (29). The results show that most PAE treatments in B6 mice cause developmental as well as behavioral deficiencies that are compatible with manifestations of FASD. Additional studies regarding timing of ethanol exposure have found exposure during the first trimester equivalent leads to decreases in cerebellar volume, while second trimester equivalent exposure leads to decreased hippocampal volume (32). The model also allows further studies on specific brain regions that may offer novel insights. The hippocampus is one of the brain regions that may be important in the understanding of the complexity of FASD phenotypes. The primary role of the hippocampus is memory consolidation (33), emphasized by hippocampal lesions leading to impaired spatial learning in mice (34). PAE leads to learning and memory deficits via changes in the hippocampus (35). Ultimately, some of the behavioral effects of PAE may be a result of molecular

changes in the hippocampus. The molecular effects of PAE have been well characterized using animal models under a plethora of conditions (various neurodevelopmental stages and different dosages of alcohol) and all of them have shown that PAE affects epigenetic and genetic processes and various neurodevelopmental pathways (36–38). A single (or in most cases, multiple) instance of alcohol exposure during fetal development can result in a lifetime of behavioral and cognitive deficits. Such results show that PAE treatments in B6 mice cause deficiencies that are comparable to the manifestations of FASD in humans.

MOLECULAR ETIOLOGY OF FASD: GENE EXPRESSION AND EPIGENETIC MARKS

The development of genomic technologies has allowed the search for molecular mechanisms underlying deficits following PAE. In both cultured neurons and *in vivo* evidence, ethanol has been shown to induce programed cell death 4 (PDCD4) protein synthesis, ultimately resulting in neuronal growth abnormalities in a rat model of PAE (39). Ethanol has also been shown to induce apoptosis via ceramide pathways, alongside stress-related kinases during development in cultured rat astrocytes (40). Direct treatment of ethanol on cultured neural stem cells often results in a host of changes at the level of gene expression. It includes *Dnmt1*, *Uhrf1*, *Ehmt1*, *Ash2 l*, *Wdr5*, and *Kdm1b* transcripts that have been shown to have significantly different levels of gene expression following ethanol exposure *in vitro* (41).

We have attempted such studies on B6 *in vivo* (42). The results show that multiple ethanol-treatment paradigms that result in FASD phenotypes also show changes in gene expression (28, 30). Such changes occur with respect to neurodevelopmental timing of exposure. More important they are representative of genomic alterations that are dependent on the biological processes occurring at the time of ethanol exposure (30, 42). Interestingly, ethanol exposure initiates alterations in a set of genes (short-term effect) that primarily affect cellular compromise and apoptosis representative of ethanol's toxic effects. In the long term, however, genes affected following PAE are very different and involve various cellular functions including epigenetic processes such as DNA methylation, histone modifications, and non-coding RNA regulation that may underlie long-term changes to gene expression patterns (43). These may be initiated by ethanol-induced alterations to DNA and histone methylation, particularly in imprinted regions of the genome, affecting transcription, which is further fine-tuned by altered microRNA (44). These processes are likely complex, genome-wide, and interrelated. The epigenetic changes may be responsible for the FASD-related alterations in gene expression. Additionally, the epigenetic changes acquired may remain stable for life and maintain the manifestation of FASD.

At least two features of this system are encouraging and offer hope for people affected with FASD and related disorders. First, human brain development is not complete at birth, rather it continues for decades. More important, the neurodevelopment during this period is rather malleable and responsive to post-natal environment. Consequently, it may provide an opportunity to direct/maneuver post-natal brain development and alter the course of development of FASD and related endophenotypes. Second, the underlying epigenetic changes brought about by PAE represent an adjustable process. Specifically, DNA methylation is known to be reversible, and may be altered using different strategies. This promise and hope offered by the two features (continuity of brain development after birth and potential to change PAE epigenetic marks) have remained poorly explored in FASD-related studies. We will present preliminary results to argue that the continuum of post-natal neurodevelopment offers an opportunity to ameliorate the effect of prenatal alcohol and adjust/restore the final outcome.

POST-NATAL ENVIRONMENT NEURODEVELOPMENT AND FUNCTIONING

Mammalian neurodevelopment is a long-lasting continuum. It begins early and finalized in early adulthood. It is also closely orchestrated and sensitive to prenatal as well as post-natal environment, particularly stresses. It makes it nimble with potential to incorporate desirable post-natal experiences. The mechanism behind this potential although recognized is not fully understood. What is known is that post-natal processes contribute to the life-long changes in behaviors and mental abilities. Also, it may result via responsiveness of the hypothalamic–pituitary–adrenal (HPA) axis (45), the primary physiological regulator of the environmental stress in mammals. Perhaps, the strongest evidence for this effect comes from post-natal handling of rodents. It involves daily separation of pups from the mother (3–15 min), a stressful event, for the first few weeks of life. Such pups show decreased stress reactivity in adulthood (46–48). Also, pups exposed to extended positive maternal care show decreased fearfulness and more modest HPA responses to stress (49, 50). Similar results have also been reported in non-human primates (51), and humans (52, 53). In each case, variations in post-natal conditions promote hippocampal synaptogenesis and spatial learning and memory through systems known to mediate experience-dependent neural development (54). The question of how post-natal environment causes such a dramatic effect in mammals has formed a fruitful area of research in recent years. It argues that this effect may be realized via the effectiveness of HPA axis.

An underlying mechanism behind the effect of post-natal environment is provided by studies by Michael Meaney and his collaborators. They found that increased licking and nursing by rat mothers altered DNA methylation of hippocampal glucocorticoid receptor in the pups. Further, the altered methylation is directly related to the development of HPA responses to stresses through tissue specific effects on gene expression (55). The results also emphasize that there is a critical period for such effects to be realized. It is particularly effective in early post-natal periods. This relationship between maternal care and gene expression via DNA methylation argues for environmental reprograming that is stable and may form the basis for the developmental origin of vulnerability to defects (56). These results have now been replicated in a number of mammals including humans. They argue that early life events can alter the methylation (epigenetic) state of relevant genomic regions, the expression of which may contribute to individual differences in the risk for pathology and diseases of fetal origin (57). Given this understanding, it is natural to consider post-natal enrichment in correction of any epigenetic pathology. It is particularly relevant in cases of FASD that are caused by

alcohol-induced alterations in DNA methylation. In fact, one may postulate potential involvement of DNA methylation (58) at every step in neurodevelopment including responses to environment prenatally as well as postnatally.

The results available have allowed us to propose alterations in the sequential continuum of neurodevelopment in FASD over a longer time frame – from fertilization to maturity (**Figure 1**). It shows the continuum of neural development with and without prenatal alcohol that result in metabolomic changes leading to either FASD or not. It recognizes that the manifestation of this outcome is not fixed. It must follow additional development and refinement in a given post-natal environment. Once again, post-natal environment may affect the developing brain via epigenetic and metabolomic alterations. We argue that such alterations will vary and depend on the nature (heavily enriched to heavily stressful) of the post-natal environment. Consequently, the effect of post-natal environment may permit recovery from prenatal effects [enriched environments (EE)] or add additional defects (stressful environment). The model covers molecular processes that underlie the initiation, progression, and completion of neurodevelopment and any role prenatal or post-natal stress may have during gestation, birth, and post-natal development. The model recognizes that the effect of post-natal environment is not restricted to cases with PAE. Rather, it is expected to have an impact on cases where there is no exposure to alcohol. Further, although the nature of prenatal stress is well defined, the nature of post-natal environment that will have a positive and negative effect remains rather generic and needs to be carefully investigated. We will discuss this model further using ongoing experiments.

ASSESSING THE POST-NATAL ENVIRONMENT ON FASD

Fetuses exposed to alcohol prenatally have poor growth in the womb. Consequently, they are born with low birth weight (59). Further, almost none of such babies have normal brain development. They also show decreased muscle tone, poor coordination, and slow growth rate (59). Naturally, newborns with FASD are dependent on post-natal care by the mother for their future development. Also, they are often born in suboptimal families and raised in suboptimal conditions, particularly in the previously outlined high-risk subpopulations. Consequently, an unfavorable post-natal environment often provides a continuation of prenatal developmental insults thereby increasing the risk and severity of the PAE outcome. It has been argued that an enriched post-natal environment may have an ameliorating effect on the brain development in the FASD babies but the effect of post-natal environment on the development of FASD phenotypes has not been adequately explored. We will assess the results of stressful and enriched post-natal environment on the growth, development, and mental ability of newborns with diagnosed with FASD.

STRESSFUL POST-NATAL ENVIRONMENT IN FASD

Results available in the literature argue that the combined effect of PAE and post-natal stress worsen the behavioral and structural effects of alcohol exposure alone (60, 61). Further accumulative stressors over time may contribute to increased risk of depression in FASD via HPA axis dysregulation (62). In humans, there are sex-differences in stress regulation, in that females show greater changes in heart rate, while males exhibit more alterations in cortisol levels (63). In primates, the combination of PAE and maternal stress led to a reduction in birth weight in males, but not females – further highlighting the sex-specificity. Additionally, both sexes show HPA axis responses following maternal separation stress (64). In rodent models of PAE, the basal levels of corticosterone and adrenocorticotropin (stress-related molecules) are unaltered, but subjects are hyper-responsive to stressors in adulthood (65). Chronic stress leads to increases in corticosterone in ethanol exposed females following an acute stress event (66), and prolonged exposure to stressors in ethanol exposed males leads to overactive HPA response (65). In B6 mice, maternal separation stress on normal pups is often used to model chronic early life stress. It uses 3 h of separation per day from post-natal days 2–14 that can result in anxiety-like behaviors in adult mice (67). The resulting mice display increased anxiety-like behaviors on open-field testing (68) similar to those observed in PAE models without maternal separation. Interestingly, behavioral abnormalities including learning and memory deficits induced by PAE and prenatal stress may be moderated by administration of BDNF (69). Further, such effects may be due to changes in hippocampal gene expression (70, 71). The general conclusion is that stressful post-natal environment may add deterioration on young's exposed to prenatal alcohols. The specific interaction between prenatal alcohol and stressful post-natal environment however, has not been sufficiently examined.

POST-NATAL ENVIRONMENTAL ENRICHMENT IN FASD

Prenatal alcohol causes FASD. Also, how prenatal alcohol may manifest the development of FASD is becoming apparent. One of the next logical questions in FASD research deals with the role of post-natal environment. Most FASD children are born into an environment of malnutrition and drug and nicotine abuse (72, 73). In addition, poor socio-economic lifestyles along-with neglectful parenting, exacerbate the behavioral and cognitive abnormalities so characteristic of FASD children. It has been hypothesized that an enriched post-natal environment may lessen the severity of the manifestation in a newborn diagnosed with FASD. An enriched post-natal environment may involve intensive physical, cognitive, and behaviorally challenging environments (74, 75). The repeated exposure to counseling sessions and specialized classes with an aim to develop verbal, math, and social skills helps ameliorate some, if not all behavioral and cognitive deficits. While some interventions manage to lessen stress and anxiety levels in FASD children, cognitive disabilities still remain at large. However, such rehabilitative therapies have been unsuccessful in improving the spectrum of ID in FASD. What is needed is a better understanding of the molecular events that follow rehabilitative therapies in humans. To this end, it will be desirable to answer the question: Do rehabilitative therapies target the very same affected molecular pathways that cause FASD or do they have different molecular mechanisms? Such questions are better explored using animal models.

What constitutes "rehabilitative therapies" in rodent models of FASD? Ethanol exposed rats and mice that are subjected to physically and cognitively challenging environments (EE) tend to be less stressed and have improved memory performance (76). Given how fetal alcohol exposure affects neurodevelopment, it is possible

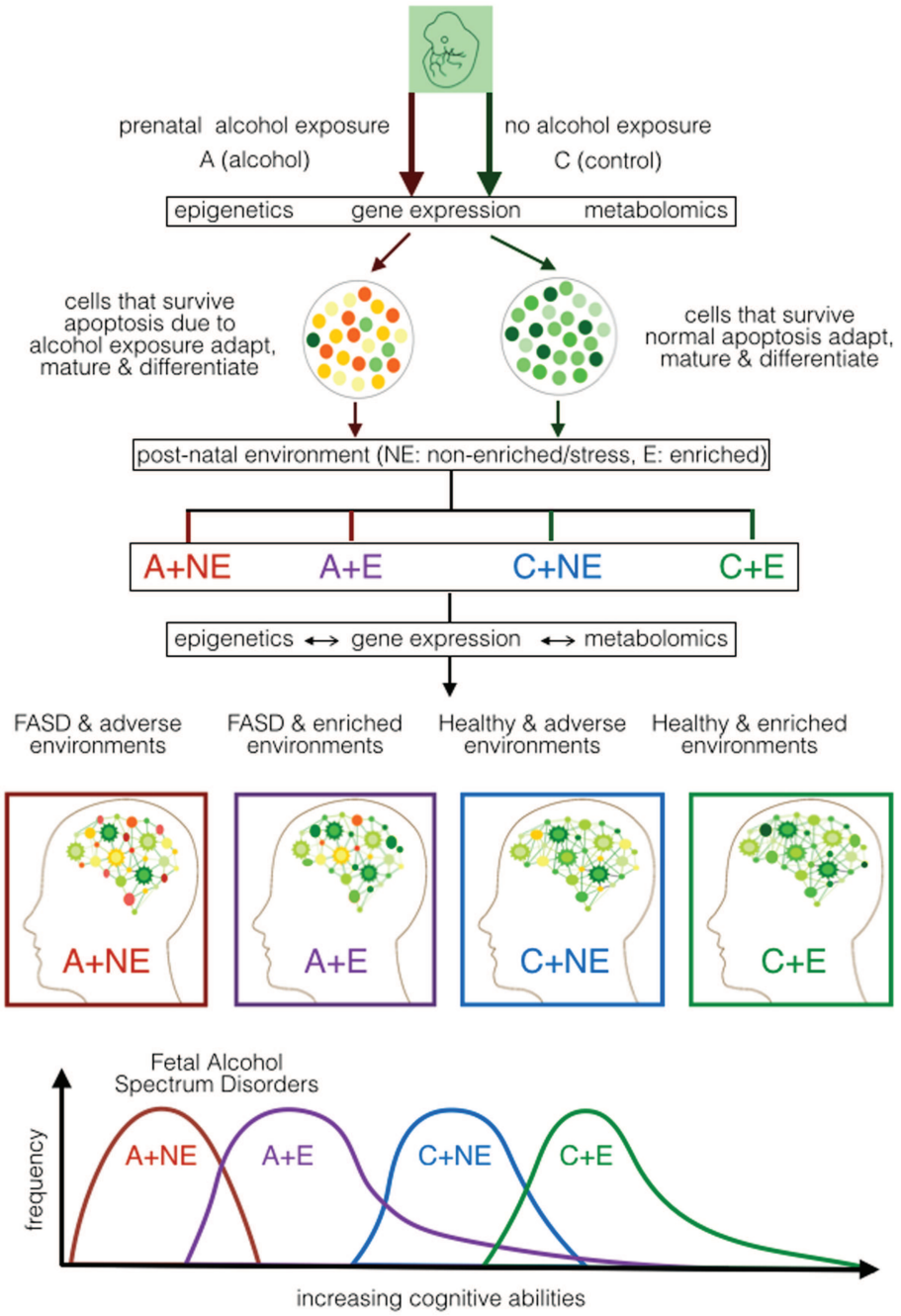

FIGURE 1 | Proposed developmental model of cognitive ability in FASD based on mouse models of prenatal alcohol exposure. It shows the effect of prenatal alcohol (A) or control (C) under normal/non-enriched (NE) and enriched (E) conditions during early post-natal development. It portrays the effect of prenatal alcohol on epigenetic, gene expression, and metabolomic alterations during neurodevelopment as well as the effect of post-natal conditions on epigenetic, gene expression, and metabolomics during post-natal development. The expected cognitive ability of the mice subjected to the four treatment combinations (A–NE, A–E, C–NE, and C–E) is projected. It suggests that the post-natal environment is capable of ameliorating (at least partly) the effect of prenatal alcohol and other effects that may affect mental ability of the newborn.

that the effectiveness of EE result from a targeted activation of specific molecular mechanisms that modify brain structure and function and are ultimately expressed as "rehabilitated" behaviors. Compared to standard housing conditions (non-enriched) with shoe-box sized cages and basic food and housing, enriched cages tend to be much larger. The latter have toys of various shapes, sizes and textures, tunnels, nesting material, heavy bedding, and access to running wheels and ladders. The objects and their locations

are changed weekly. Such environments facilitate mice to burrow, climb, chew, run, and explore new objects and placements, thereby engaging and developing cognitive processes. To eliminate stress due to isolation and or lack of social interaction, all mice, whether in standard or enriched cages are socially housed.

Our lab, amongst others has been interested in learning more about the effects of a positive, enriched post-natal environment on mice exposed to alcohol prenatally. Our first objective has been to demonstrate that environmental enrichment can ameliorate some, if not all of the behavioral and cognitive deficits that are characteristic FASD phenotypes. Four groups of mice have been generated: Control/Saline (C) mice living in enriched (CE) and non-enriched (CNE) conditions and prenatal alcohol exposed mice (A) living in enriched (AE) and non-enriched (ANE) conditions. Our results show that FASD mice that have been exposed to environmental enrichment (i) exhibit a fewer number of anxiety-like traits (as evidenced by more time spent in the light-region of the light–dark box and open-arms of the elevated-plus maze) and (ii) perform relatively better in learning and memory tests (as evaluated by the novel-object recognition and the Barnes maze). This experimental design has also allowed us to establish that enrichment not only ameliorates behavioral and cognitive deficits of affected mice (AE versus ANE) but improves these characteristics in normal, healthy control mice that had never been exposed to alcohol (CE versus CNE). Group comparison has also shown that prenatal ethanol exposure causes permanent and long-lasting damage to the developing brain. Further, the post-natal environmental enrichment is successful in ameliorating these deficits only to a certain extent. The mechanism involved in this amelioration is poorly understood and deserves further research.

The long-lasting effects of environmental enrichment have implicated changes in epigenetic machinery. Such results in conjunction with other lines of evidence show that the DNA methyltransferases (DNMTs) and histone acetyltransferases (HATs) are essential in neurodevelopment activities such as neural stem cell proliferation, differentiation, and synaptic plasticity (77–79). Work by Rampon et al. was among the first to show that DNMTs are preferentially up-regulated in the brains of healthy mice that have undergone environmental enrichment (80). While a number of genes involved in neuronal structure, neural plasticity, and synaptic signaling were up-regulated, the highest levels of induction was found in DNMTs. These enzymes are critical in neural cell differentiation induced by nerve growth factors (80). In 2011, Lopez-Atalaya et al. investigated the role of the histone acetyltransferase CREB-binding protein (CBP) in the context of environmental enrichment (81). CBP has been shown to be involved in neural plasticity and memory processes in the brain. Dysregulation of CBP is associated with a complex epigenetic disorder known as Rubinstein–Taybi syndrome, characterized by behavioral and cognitive deficits. CBP-deficient mice undergoing environmental enrichment have ameliorated physiological and behavioral deficits. In addition, multiple roles of CBP in neurogenesis and neuroadaptation to environmental changes were identified (81). Environmental enrichment has been shown to cause a dramatic increase in IDNA levels of BDNF, with concomitant widespread changes in histone methylation at various BDNF promoters and no change in the expression levels of several brain-specific microRNAs (82). Various other studies have pointed out the important role of BDNF in learning and memory processes (83, 84), particularly how BDNF shapes the cognitive and stress-response trajectory of neurodevelopment through interactions with the HPA axis (85–88). Our lab and others are currently investigating the effects of environmental enrichment on mice following alcohol exposure in the context of BDNF and its associated epigenetic marks to gain a better understanding of how the post-natal environment acts to ameliorate negative phenotypic outcomes as a result of alcohol exposure.

SYNTHESIS AND FUTURE PERSPECTIVE

Most intellectual disabilities in children are caused by neurodevelopmental aberrations. Often they involve complex interactions of genes and environment over prenatal and post-natal periods. For example, intellectual disabilities in FASD are caused by PAE that disrupts neurodevelopment via alterations in gene expression. This affects a number of pathways that undergo changes during ontogeny over time. Here, the primary effect of alcohol covers cellular compromise and apoptosis, the expected toxic effect of ethanol. It leaves a molecular footprint that is shared among neurological disorders. The genes affected are related via hub molecules. More important, these results may last for life. We attribute them to epigenetic changes. The epigenetic machinery affected includes DNA methylation, miRNA, and histone modifications (44). The results argue that epigenetic features are critical during neurodevelopment. Any aberration in ongoing epigenetic marks at any stage during neurodevelopment may result in intellectual disabilities. It follows reports that have implicated epigenetic causes in intellectual disabilities (89–91). Such a conclusion has far reaching implications including prospect for an epigenetic therapy (92). We anticipate that this will be a major challenge for the scientific community in the next decade.

We argue that relatively long time course of neurodevelopment offers an opportunity to apply potential epigenetic therapy in intellectual disabilities. For example, a prenatal defect may be corrected following birth during early post-natal development. At this stage, developing brain is malleable. Also, it is responsive to variety of mediators including drugs, care, and social interactions. As stated, it is possible to partially ameliorate FASD deficits by post-natal environmental enrichment in B6 mice. The current most logical mediator for any amelioration in humans appears to be the early environment enrichment including cognitive therapy and interactive schooling. It is considered most logical and effective.

Rodent research also suggests that animals raised under environmentally enriched conditions exhibit relatively fewer stress- and anxiety-like traits. Also, they have improved learning and memory. Further, just like the effect of prenatal alcohol, the recovery of FASD-related cognitive dysfunction due to post-natal environment also involves epigenetic processes. Such results are encouraging for the reversal of epigenetic marks. Although the specific methods for this reversal are not apparent, rehabilitative therapies and drug regimes that target epigenetic pathways would provide a good starting point. To this end, the further research should clarify two aspects of this research. First, what is the relationship between DNA methylation, histone modification and microRNA expression, brain structure and function,

and intellectual ability including intellectual deficits in the FASD model? Second, what are the genetic pathways and mechanisms that might be targeted in future attempts to treat behavioral, cognitive, and intellectual deficits associated with human fetal alcohol exposure? The answer to such questions will have the potential to identify suitable treatments for ID caused by neurodevelopmental aberrations.

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A comparison of the different animal models of fetal alcohol spectrum disorders and their use in studying complex behaviors

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Prenatal ethanol exposure (PNEE) has been linked to widespread impairments in brain structure and function. There are a number of animal models that are used to study the structural and functional deficits caused by PNEE, including, but not limited to invertebrates, fish, rodents, and non-human primates. Animal models enable a researcher to control important variables such as the route of ethanol administration, as well as the timing, frequency and amount of ethanol exposure. Each animal model and system of exposure has its place, depending on the research question being undertaken. In this review, we will examine the different routes of ethanol administration and the various animal models of fetal alcohol spectrum disorders (FASD) that are commonly used in research, emphasizing their strengths and limitations. We will also present an up-to-date summary on the effects of prenatal/neonatal ethanol exposure on behavior across the lifespan, focusing on learning and memory, olfaction, social, executive, and motor functions. Special emphasis will be placed where the various animal models best represent deficits observed in the human condition and offer a viable test bed to examine potential therapeutics for human beings with FASD.

Keywords: FASD, behavior, animal models, alcohol, prenatal ethanol exposure

INTRODUCTION

Ethanol is a teratogen that disrupts normal development. The use of animal models to study how ethanol affects the development of offspring in animal models can be traced back to the late 1970s, when several groups began to study ethanol's effects on the development of laboratory rats. How this agent affects the development of the brain and body remains a subject of intense investigation, and it is worthwhile to appreciate some of the guiding principles that drive this field of investigation, particularly as they relate to the choice of animal model to be used. The choice of animal to be used can be critical, as genetic susceptibility can play a major role in determining ethanol's effects. For instance, in some species any teratogenic effects may be induced with relatively low doses, while other species may be more impervious to the effects of ethanol. Second, one has to appreciate that the developmental stage of the organism at the time of exposure can play a significant role in how ethanol disrupts development. There are critical periods of *in utero* growth and development where certain brain or organ systems will be undergoing rapid development and thus be more prone to damage by teratogenic agents. Third, understanding how teratogenic agents act on proteins and signaling systems in developing cells will be key to understand how ethanol can initiate sequences of abnormal development at a cellular level. Certain animal model systems will lend themselves more readily to these sorts of experiments, depending on the nature and complexity of the question being asked. Fourth, it is critical to understand the nature of the agent itself, as the route and degree of maternal exposure, as well as the rate of placental transfer and systemic absorption are key factors in determining how severely ethanol will affect organism. A fifth consideration is that one needs to be vigilant for the four major signs of deviant development (death, malformation, growth retardation, and functional defect) when examining the effects of ethanol in any animal model. Functional defects may occur without any significant malformation or growth retardation. Finally, it should be clear that any disruptions in normal development will likely increase in frequency and degree as dosage increases. Ethanol is unusual in that it is both lipid and water soluble, so when it is consumed by pregnant females it can rapidly transit the placental membrane and directly affect the fetus (1). With these considerations in mind, let us briefly examine what we know of how ethanol affects the human condition.

Fetal alcohol syndrome (FAS) is the most severe disorder that results from prenatal ethanol exposure (PNEE). FAS is

Abbreviations: ARBD, alcohol-related brain defects; ARND, alcohol-related neurological disorder; BAC, blood alcohol content; CNS, central nervous system; EDC, ethanol-derived calories; FAS, fetal alcohol syndrome; FASD, fetal alcohol spectrum disorder; GD, gestational day; i.p., intraperitoneal; IQ, intelligence quotient; MWM, morris water maze; PC, Purkinje cell; PND, postnatal day; PNEE, prenatal ethanol exposure; s.c., subcutaneous.

a disorder characterized by facial dysmorphologies (such as midfacial hypoplasia, wide spaced eyes, and a smooth philtrum), growth retardation, and CNS dysfunction resulting in cognitive, motor, and behavioral problems (2). Since FAS was first defined in the 1970s (3, 4) researchers have become more aware that the damage caused by ethanol can vary due to the timing, frequency, and volume of ethanol consumed. In addition, genetics and the metabolism of the mother can also play a role (5), leading to significant variability in the severity and symptoms associated with PNEE. Understanding that variability in genetic make-up, and variability in the timing and dose of ethanol consumption, can impact how ethanol affects development has resulted in the umbrella term FASD being adopted to refer to any condition that results from PNEE. This term encompasses children who exhibit varying degrees of central nervous system (CNS) dysfunction including alcohol-related birth defects (ARBD) and alcohol-related neurological disorders (ARND) that result from PNEE. These conditions often lack the facial dysmorphology needed to meet the diagnostic criteria for FAS, but are never-the-less the result of exposure to this teratogen during development (2, 6).

Although we have been aware that ethanol is a teratogen since the 1970s, there are still large numbers of children affected by PNEE (7). In part, this is because many women do not realize they are pregnant in the first trimester and continue binge drinking (8, 9). Furthermore, in many countries a significant percentage of pregnant women continue to consume ethanol throughout pregnancy – 10–20% in the USA, 40% in Uruguay, and 50% in some parts of Italy (10–12). In the United States, the lifetime cost for an individual suffering from FAS may be as high as $2 million. The majority of these costs are required for special education, medical, and mental health treatment (13). Currently in Canada, the annual cost of health care problems associated with PNEE is over $5 billion (14).

COGNITIVE SYMPTOMS

Prenatal ethanol exposure can lead to a host of cognitive impairments. The severity and nature of these impairments depends on the amount and duration of alcohol consumption during pregnancy (4, 15–19). Children with FASD display a multitude of neuropsychological issues including deficits in mathematical ability, verbal fluency, memory, attention, learning capabilities, executive function, fine motor control, and social interaction, with the number of issues and the extent of damage varying from child to child (15, 17, 19, 20). To be diagnosed with an intellectual disability, generally a child must have an intelligence quotient (IQ) two or more standard deviations below the norm, roughly equating a score below 70, while scores between 71 and 85 are considered to represent borderline intellectual function [DSM V (21)]. Children with FAS generally have IQs estimated in the low 70s but the range can be anywhere between 20 and 120 (16, 22). Children without the complete FAS diagnosis (but with the FASD diagnosis) also generally have low IQs with averages in the low 80s (23).

UNDERLYING MECHANISMS OF PNEE DAMAGE

Because of the variety of deficits that occur with FASD it can be hard to pinpoint the structural and functional changes that occur in the developing CNS and to identify how they relate to a particular behavioral disorder. Multiple brain regions are affected, and the areas and extent of damage depend on the amount and timing of ethanol ingestion. A number of molecular mechanisms may play a role, and these may be activated at different stages of development or at different dose thresholds of exposure [see Ref. (24, 25) for review]. These include disrupted cell energetics (26–30); cell cycle interference, and a deregulation of developmental timing (31–35); alterations in retinoic acid signaling (36); interference with cell and growth factor signaling (37–39); and apoptosis (38, 40, 41). Furthermore, many neurotransmitters, adhesive molecules, transcription factors, and trophic factors can be either up- or down-regulated by PNEE, making FASD a very complex syndrome [see Ref. (24) for review].

OBJECTIVES

The study of human subjects is invaluable for FASD research, however, epidemiological studies are often limited by ethical constraints and a multitude of confounding variables including multi-substance abuse, diet, maternal health, and genetic or socioeconomic background (25, 42). It is also difficult to get reliable estimates on the amount and timing of ethanol exposure when self-reporting from the mothers is necessary. Due to these constraints, studies in human beings have focused on finding biomarkers of PNEE in fetal meconium (43) and hair samples [see Ref. (44) for review] through the presence of fatty acid esters [see Ref. (45) for review].

Animal models provide a simple and reliable method to study the effects of alcohol on the developing brain and eliminate many of the obvious confounds associated with human studies. These models can be used to understand the mechanism of the toxic effects of ethanol on the developing brain and to develop and test potential therapies to combat these effects. Animal models enable the experimenter to manipulate social and behavioral contexts; to control for stress and nutritional variables; and to do all of this in an organism that has a condensed lifespan in relation to human beings. In this review, the different animal models of FASD will be outlined and the advantages and disadvantages of each model will be discussed. This will be followed by an in depth discussion of the cognitive deficits that have been observed in the animal models of PNEE.

FACTORS TO CONSIDER WHEN MODELING FASD

Because FASD is such a complex disorder and there are so many facets to explore, there are many factors to consider when choosing an appropriate model for a particular study. The level of intoxication achieved during brain development, the particular period of brain development that is to be targeted (first, second, or third trimester), the pattern of administration (chronic or acute) and the route of administration (ingestion, injection or inhalation) can all be manipulated.

There are also a wide variety of animal models available for FASD research ranging from the simple (*Caenorhabditis elegans*, *Drosophila*, zebrafish, *Xenopus*) to the complex (rodents and non-human primates). Rodents are by far the most common model employed, with rat, mouse, and guinea pig models utilized in laboratories throughout Canada and the USA. All these models have been shown to mimic at least some aspects of the

human condition including the craniofacial abnormalities (46, 47), growth retardation (48–50), physiological impairments (51–53), and cognitive deficits (42, 54–56) reviewed in Ref. (42, 57). However, similar to the variability that is observed in human beings, there is no single animal model that mimics all the features of FAS and/or FASD. When deciding on which model to utilize, it is pertinent to choose based on the research question to be examined. In this section, we will first discuss the pertinent factors to consider when designing a study of PNEE followed by a breakdown of each of the animal models, with the major strengths and limitations of each method considered. It is important to note that we have limited our discussion of animal models to simple systems (*C. elegans*, *Xenopus*, and zebrafish) and more sophisticated rodent and non-human primate models. There is also FASD research being conducted using chicken (58) and sheep (59–62) models, however, because there is little behavioral analysis using these models we have omitted them from our review.

BLOOD ALCOHOL CONCENTRATION

In Canada and USA, a blood alcohol concentration (BAC) of 80 mg/dl is considered legally intoxicated. If a 150 lb pregnant female consumes six alcoholic beverages, or a bottle of wine in a 2 h period a BAC of 200 mg/dl would be reached. In human studies, the BAC data from the mothers are generally not available, however, estimates suggest that BACs of over 200 mg/dl may be responsible for the severe FAS phenotype (63), while lower BACs may produce milder forms of FASD. Despite the lack of BAC data in human beings this measure is often used to compare exposure levels across species. This is because the absolute dose of ethanol administered (in gram of ethanol/kilogram) can vary greatly from species to species (42) so the BAC is a more reliable measure of intoxication.

Most animal studies use a dosage of alcohol exposure that produces a BAC in the range of 100–400 mg/dl (i.e., moderate to binge-like levels of exposure). The peak BAC achieved will depend on both the dose and pattern of exposure (64, 65). In order to achieve a low to moderate BAC (80–150 mg/dl), experimenters normally employ either liquid diets, voluntary drinking paradigms, or vapor inhalation (see Route of Administration). Higher binge-like BACs (>200 mg/dl) are normally achieved using either oral intubation (gavage) or direct injections (see Route of Administration). Higher BACs are generally associated with increased neurotoxicity, and even the administration of a single high dose of ethanol during the period of brain development can cause significant structural impairments if the BAC achieved is sufficiently high (66, 67). Low to moderate BACs can also cause significant neuronal damage, and while longer exposure periods (i.e., throughout gestation) are usually used with these models (68–70), shorter exposure can still cause significant deficits (31). Thus, continuous low-level exposure to ethanol may be as damaging as a single high-level exposure, though the types of deficits incurred may differ. The deficits observed with either mode of administration can be affected by the timing of ethanol exposure.

DEVELOPMENTAL TIMING OF ETHANOL EXPOSURE

The timing of ethanol exposure can greatly influence the outcome of the fetus. The mammalian brain develops in six major phases, commencing with neural cell genesis, followed by neuronal migration, glial cell proliferation, axon and dendrite proliferation, synaptogenesis, extensive pruning and cell death, and finally myelination of the axons (71). These steps occur in all regions of the brain but different regions develop at different times depending on their caudal or rostral location. Brain development is a dynamic process and it is therefore important to consider the developmental timing of alcohol exposure when choosing a model, based on regional and temporal windows of vulnerability. Gestation and development in simple vertebrates (e.g., *Xenopus*, *C. elegans*, or zebrafish) and even rodents (mice, rats, guinea pigs) is significantly different from human beings. The human gestation period is characterized by three trimesters, all of which occur prenatally. In the first trimester, formation of the neural tube and gastrulation occurs and in the second trimester cell proliferation and migration occur profusely. Finally, in the third trimester a "brain growth spurt" occurs, which is characterized by large amounts of growth and differentiation (72).

Rodents are the most commonly used animal model used for FASD research (see Rodents), however, their gestational period is much shorter than that of human beings (18–23 days for mice/rats; 68 days for guinea pigs), and a significant amount of brain development occurs following birth in these species (73, 74). The development period of the rodent brain is also divided into trimester equivalents; in the guinea pig, the three trimester equivalents largely occur prenatally, and therefore more closely resemble brain development in human beings. In rats and mice, the first trimester equivalent is from gestational day (GD) 1–10, the second trimester equivalent corresponds to GD 10–20 (just prior to birth) and the third trimester equivalent and "brain growth spurt" occurs following birth [from postnatal day (PND) 1 to 10] (75). In order to expose the brain to alcohol through all three trimester equivalents, alcohol must be administered to neonate pups (via oral intubation; see Ingestion), and the mechanisms of exposure, absorption, and elimination of this substance are significantly different during the prenatal and postnatal periods. For example, ethanol metabolizing enzymes, such as alcohol dehydrogenase, are only at 25% of adult levels at birth (76). Normally, the fetus is partially protected by the mothers' capacity to metabolize ethanol, so in rodent pups it is routinely reported that higher BAC levels are produced in neonates with lower alcohol doses than those used in pregnant dams (77–80).

ROUTE OF ADMINISTRATION

There are several different methods that can be used to administer ethanol during pregnancy. In invertebrates and simple vertebrates (*C. elegans*, *Xenopus*, zebrafish), alcohol exposure is usually by bath application (see Simple Systems). In more complex models such as those using rodents and primates, there are three major methods of ethanol administration employed: ingestion (through diet, water, or intubation), injection, or inhalation [for additional reviews see Ref. (25, 81, 82)].

Ingestion

Dietary. The liquid diet model of ethanol exposure is one of the most commonly utilized routes of delivery in mouse and rat models and was one of the first models to be developed (83–85).

Generally, food is provided to pregnant dams as a liquid diet throughout gestation in which a percentage of the calories (usually ~35%, which equates to 6.61% v/v) are derived from ethanol (**Figure 1**). This diet is the only source of nutrition throughout the pregnancy. Using this method, rats can consume on average 12 g ethanol/kg/day (and up to 18 g/kg/day) (25). Consumption of the diet usually begins on GD 1 of pregnancy, and the diet is introduced slowly over a three-day period (i.e., one third final ethanol concentration on GD1, two thirds of final ethanol concentration on GD2, and final ethanol concentration on GD3 and for the remainder of the pregnancy). Pair-fed control groups are often included when using this method, where an isovolumetric, isocaloric replacement (such as maltose dextrin) for the ethanol calories is used and food is restricted to that of the ethanol consumption groups (86) (see Finding the Right Control Group). The liquid diet model reliably produces BACs between 80 and 180 mg/dl in rats, i.e., a low to moderate level of exposure (15, 17, 55, 87–90), which are accompanied by neurological deficits similar to what are observed in children with FASD (see Blood Alcohol Concentration).

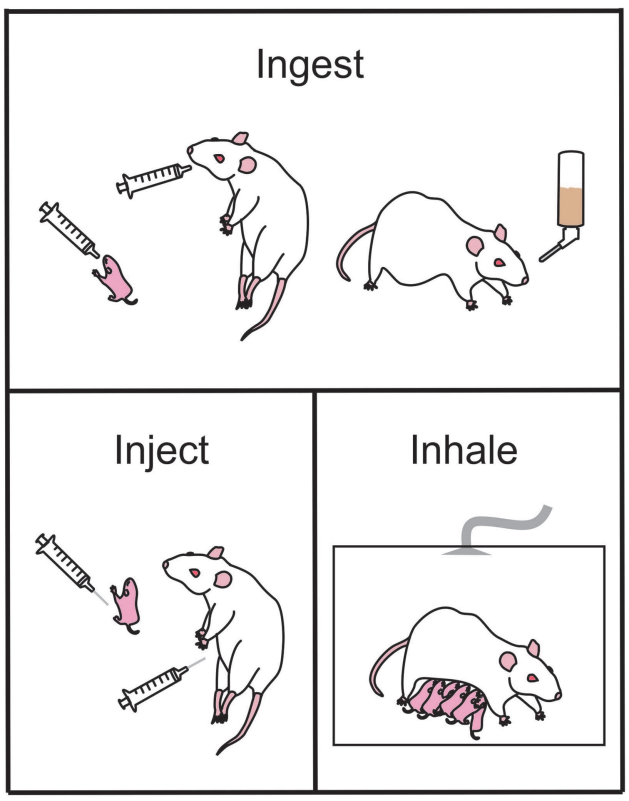

FIGURE 1 | Common ethanol administration techniques in rodents used to examine the effects of prenatal ethanol exposure in offspring. Ethanol may be ingested by the animal via gavage administration during the early postnatal period (upper panel, left-most) or during gestation (upper panel, middle). Alternately, ethanol may be ingested as a liquid diet (upper panel, right-most). Ethanol injections (bottom left panel) can be administered pre- or postnatal for studies of exact timing of ethanol-induced damage. During the early postnatal period of offspring, the dam and litter can be placed in vapor chambers and be exposed to inhaled gaseous ethanol (bottom right panel).

Voluntary drinking. Similar to the liquid diet model, ethanol can also be administered through the drinking water (**Figure 1**). This is usually achieved by training female mice or rats to voluntarily consume a saccharin-sweetened 10% ethanol solution prior to pregnancy (68, 91). Control groups receive saccharin-sweetened water only. Throughout pregnancy the rodents have *ad libitum* access to regular rat chow. Following birth, ethanol is removed from the water in a step-wise fashion to prevent ethanol withdrawal effects (68, 91). Using this paradigm, rodents tend to consume 14 g ethanol/kg/day and the BAC achieved is 120 mg/dl (68, 91).

Advantages of the liquid diet or voluntary drinking models are that the techniques are simple, less time consuming, and less labor intensive when compared to other methods. There is also much less handling of the animals associated with these procedures (a source of stress) and there is less risk of fatality. Disadvantages result because this method does not allow for the precise control over dosage or timing of ethanol exposure and this can lead to increased variability in the BAC achieved, as ethanol consumption depends on voluntary food consumption throughout the day. For example, a study by Mankes et al. (92) found that ethanol consumption of a group of 221 rats fed a liquid diet ranged anywhere between 4 and 18 g/kg/day depending on that rat (92). It is also important to remember that the liquid diet or voluntary drinking paradigms do not include alcohol exposure during the third trimester equivalent. While pregnant dams could be continued on a liquid diet during the suckling period, it is uncertain how much ethanol can cross into the breast milk and the actual dose of ethanol consumed by the pups could not be controlled for. Dams consuming ethanol during the suckling period may also be less attentive to their pups and may not engage in appropriate maternal behavior leading to social and nutritional stress [see Ref. (25) for review]. Therefore, these models are normally only used to examine exposure during the first and second trimester equivalents in the rat and mouse. Because human mothers can often be unaware they are pregnant and inadvertently drink during these periods, these models still have significant legitimacy for the human condition.

Intragastric intubation (gavage). Ethanol can also be delivered directly to the stomach using an intubation method (73, 74, 78–80, 93, 94). Typically, a syringe is attached to a curved steel gavage needle, or plastic tubing, that is inserted down the esophagus to the entrance to the stomach (**Figure 1**). This method allows ethanol to be administered to pregnant females (ethanol is usually diluted in water or saline) and to neonatal pups (ethanol is usually diluted in a nutritional formula). An isocaloric control liquid (such as maltose dextrin or sucrose) can also be administered by gavage to control for the stress and nutritional effects of this procedure. This method is commonly used in rodents including rats (74, 78–81, 95–98) and guinea pigs (99–103), as well as primates (104–107). The dose of ethanol typically ranges between 2 and 6 g ethanol/kg/day; but produces BACs generally greater than 200 mg/dl. Often the daily dose of ethanol is divided into two administrations, given 4–8 h apart, creating two lower peak BACs in a 24 h period (42). A major

advantage to this method of administration is the precise control over the dose administered and hence the peak BAC reached. A further advantage is that neonatal pups can be exposed to ethanol, allowing study of the effects of ethanol during the third trimester "brain growth spurt." However, care must be taken to ensure that neonates adequately gain weight during the period of alcohol consumption and often a milk supplement needs to be provided to maintain healthy body weight [see Ref. (25) for review]. A significant disadvantage of intragastric intubation is that it is invasive and a very time-consuming procedure to undertake. Increased stress and higher mortality rates are also associated with this model, and individuals performing this procedure need to undergo specific training to become competent in the procedure.

Artificial rearing (pup in a cup). In order to provide neonate rodents pups ethanol during the third trimester equivalent, pups can be reared artificially though a method colloquially known as "pup-in-the-cup" [see Ref. (25) for a review]. In this procedure, the pup receives intragastric ethanol, or a control solution while being maintained in a warm cup filled with nesting material in an effort to mimic the cage environment and maternal interaction early in life (108, 109). Although this method can be used to reliably administer known amounts of food and ethanol, it is invasive, expensive, and isolates each pup, removing many of the social factors that are present during normal neonatal development (i.e., presence of littermates, maternal grooming, etc.).

Injection

Ethanol is often administered to rodents via a subcutaneous (s.c.) (40, 110, 111) or intraperitoneal (i.p.) injection (112–114) either acutely or across multiple days during gestation (**Figure 1**). This method of administration is particularly useful for examining the acute effects of ethanol on distinct periods of development, and allows for a rapid increase in BAC with limited handling-induced stress. However, this method of administration does not resemble ethanol consumption in human beings and may not accurately replicate several important aspects of human PNEE. For example, i.p. injections of ethanol during the first trimester equivalent in mice result in a higher incidence of malformation when compared to the same ethanol dose delivered via intubation (114). Ethanol administered i.p. to pregnant guinea pigs was also shown to cross from the intraperitoneal space into the uterus and chorioamniotic membranes and amniotic fluid as well as being absorbed into the mothers circulation (115). This indicates that the fetus is exposed to high levels of ethanol very soon after injection, which does not accurately mimic what occurs following oral ingestion.

Inhalation

The inhalation mode of administration is not as commonly used as some of the other methods but a brief overview of the procedures is warranted for this review. Using this method, pregnant dams, neonatal pups, or the dam and her litter are placed in an inhalation chamber filled with ethanol vapor for several hours (116–119) (**Figure 1**). This method causes a rapid, reliable increase in BAC without the stress of intubation. It is also much less labor intensive than other methods and multiple animals can be in the chamber at one time. However, this method of administration does not mimic the route of intake in human beings and therefore may not be an accurate model of FASD. Additionally, the irritation to the upper respiratory tract by vaporized ethanol can be a significant factor to consider. If this method is used to expose rat or mouse pups to ethanol during the third trimester equivalent, then pups may have to be removed from their mothers for extended periods of time that may result in reduced food intake and stress associated with the separation (117), which can have lifelong effects on pups (120, 121). Finally, this method does not currently have an effective control group to account for the loss of nutrition and separation stress in the newborn pups.

Choosing an administration model

When deciding on the appropriate route of administration, the first issue that should be considered is the BAC we want to achieve. The easiest way to get high binge-like BACs is to inject ethanol. Using this method, stable high BACs are achieved in 45 min to 1 h following injection (111). Oral intubation with ethanol or an ethanol/milk mix can also produce high BACs with maximal effects 2 h post-injection (79, 80). The benefit of the oral intubation route of administration is that it is resembles the human condition – the ethanol is being consumed orally, and therefore enters the circulation through the same mechanisms through which it occurs when a human beings consumes alcohol. If moderate steady BACs are more relevant to the research question, then choosing a liquid diet or voluntary drinking model is more appropriate, as BACs between 80 and 180 mg/dl are usually achieved (55, 88–90, 122–128). However, there is more variability associated with this model, because an animal's eating patterns may differ throughout the day and through each day of the pregnancy.

Another issue which needs to be considered when using many of the well established models of FASD is that ethanol is often given chronically (i.e., via a liquid diet or oral intubation) throughout gestation. This method of administration may not directly resemble the human condition. Pregnant human females are more likely to binge drink early in the first trimester, prior to discovering they are pregnant, or drink moderately on a couple of occasions each month throughout pregnancy (129). While the period of liquid diet exposure or oral intubation can be restricted, this often introduces large amounts of variability into the groups, which can make it difficult to infer the direct effects of ethanol. For example, in a recent study both the liquid diet and gavage models were utilized to expose rats to ethanol during the first (liquid diet), second (liquid diet), or third (gavage) trimester equivalent. When synaptic plasticity in the hippocampus was examined in adult animals, the variability between models was significant enough to mask differences caused by ethanol alone between the treatment groups (130).

FINDING THE RIGHT CONTROL GROUP

As well as affecting the brain, alcohol can also irritate the gut and can affect nutrient intake and absorption (131). In fact, it can often be difficult to separate the nutritional effects that accompany alcohol consumption from the teratogenic effects of alcohol alone (131, 132), and some studies suggest that nutritional deficits exacerbate the effects of alcohol (133–135) or that supplementation during the period of alcohol exposure may limit damage

(131, 136). Because of the large interplay between alcohol and nutrition, having appropriate nutrition controls that help to distinguish between the deficits due to diet and the deficits purely due to the teratogenicity of alcohol are important to consider when choosing a model. A "pair-fed" control is often utilized for this purpose in most rodent models of FASD. A pair-fed group acts as a calorie-matched control group, with each animal receiving the same amount of food in g/kg/day as its matched ethanol consuming pregnant dam. Normally, a carbohydrate substance (such as maltose dextrin or sucrose) is used to account for the ethanol-derived calories in the diet. Using a pair-fed group can also control for the stress of any procedures that the ethanol group may be subjected to. For example, if an oral intubation method of ethanol administration is used, pair-fed animals can be orally intubated with an isocaloric amount of maltose dextrin/sucrose, and are therefore subjected to the same procedures as the ethanol animals.

While the use of a pair-fed control group is desirable, it should also be noted that they are not a perfect control group. They are required because animals receiving ethanol generally consume less food (and therefore less calories) than control animals (79, 90, 124, 126). However, imposing caloric restriction on naïve animals can also be perceived as introducing a stressor which can be a confounding factor in many studies (69, 124, 137). While ethanol-exposed animals eat less food voluntarily, pair-fed animals are forced to eat less and spend many hours of the day hungry. Another potential problem with this model is that ethanol has inflammatory effects in the stomach [see Ref. (138) for review]. This means that any food that is ingested may not be metabolized as efficiently, and nutrients from the food that is consumed may not be absorbed (139, 140). Unfortunately, this side-effect of ethanol consumption cannot be replicated in pair-fed animals, and therefore it is not possible to be entirely certain that the results observed are not due to a lack of absorption of nutrients. However, in mothers consuming ethanol during pregnancy, this mal-absorption would also occur, therefore the effects we see are reflective of what occurs in alcohol consuming mothers.

Rodent models using the liquid diet model of ethanol exposure, where pair-fed animals consume a liquid diet with maltose dextrin substituted for the ethanol-derived calories, show varying results in pair-fed animals, with some studies showing deficits (128, 141, 142), and others showing no differences between pair-fed animals and controls (88, 143, 144).

SPECIFIC ANIMAL MODELS OF FASD

Simple systems

There are several invertebrate species that have been employed for alcohol studies. For FASD research, the microscopic nematode worm *C. elegans* is the most commonly used. While mammals offer significant advantages over invertebrates when examining brain structures or complex behaviors, simple invertebrates such as *C. elegans* can be extremely useful when examining basic biological development at the cellular, molecular, and genetic levels (145). The complete genome of *C. elegans* has been sequenced, and the simple nervous system contains only 302 neurons with 5000 synapses. Furthermore, the stages and timing of embryonic development are well characterized and a transparent egg allows for direct visualization of each of the developmental stages. A significant disadvantage to using this model is that the egg develops outside of the body and therefore alcohol exposure cannot occur as it does in human beings (via the placental membrane following oral ingestion). Instead, *C. elegans* eggs or newly hatched larvae are exposed to ethanol through bath application (145). Another disadvantage with this model is that BACs cannot be directly measured. However, if ethanol is applied at a 0.4 M concentration, previous studies in adult *C. elegans* have shown that an internal ethanol concentration equivalent to 100 mg/dl can be reached (146). In studies that have used this model to examine the effects of ethanol on development, ethanol exposure produced, in a dose-dependent manner, significant growth retardation, slowed the developmental process, impaired reproduction, and lead to early demise in the offspring (145, 147) indicating that ethanol can have similar effects on development in *C. elegans* as in human beings. Future work using this model may be able to shed light into some of the genetic mechanisms of PNEE, and whether particular genes may confer sensitivity or resistance to the toxic effects of ethanol during development (145, 147).

Simple vertebrates such as the zebrafish (*Danio rerio)* and the clawed frog (*Xenopus laevis*) are also commonly used in scientific research. These animals are cheap, small, easy to keep, have a very short developmental period, and can produce large amounts of offspring (148). Like *C. elegans*, early stage embryos have a transparent egg, and the mature zebrafish or immature *Xenopus* tadpole are also relatively transparent, allowing internal structures to be imaged very easily. Because the stages of development are thoroughly understood and can be visualized easily, it is possible to expose embryos to ethanol during very distinct and short periods of development, which can be very important for determining critical periods of ethanol exposure (149).

Also important for FASD research is the fact that the genomes of these simple vertebrates are completely sequenced and many of the genes have a mammalian counterpart. However, like with *C. elegans*, the developmental process and the physiology between these species and a human are very different. An advantage of using simple vertebrates over invertebrates such as *C. elegans* is that simple behaviors can be tested in both zebrafish and clawed frogs. This means that these animals can be used to assess functional deficits following PNEE as well as anatomical or physiological deficits (148). However, ethanol application using these organisms must still occur through bath application, with ethanol having to infiltrate the chorion of the egg, so actual concentrations of ethanol that the embryo is exposed to can be highly variable and large doses of ethanol are needed to ensure that adequate amounts cross into the embryo (binge-like exposure) (150, 151).

Studies utilizing the zebrafish or clawed frog as models for PNEE have shown that ethanol exposure during development can cause growth retardation including reduced body length, microcephaly, skeletal deficits, and eye malformation (48, 149–154) as well as cognitive dysfunction in simple behavioral tasks such as visual acuity tests (149), associative learning (54), and social behavior (155), which were apparent even in the absence of physical malformations (54, 155). These deficits were also accompanied by changes in gene expression (151, 153, 154). These effects were dependent on the dose of ethanol used and the developmental

timing and length (chronic vs. acute) of exposure, with the blastula, gastrulation, and somitogenesis periods being particularly sensitive to the effects of ethanol (48, 150).

Rodents

Rodents are the most commonly employed models for FASD research. Rodent models are ideal for exploring basic science questions that relate to molecular biology, synaptic plasticity, and cognition. There is also a vast body of literature on rodent physiology, behavior, anatomy, development, reproduction, and teratology (25, 57). The advantages and disadvantages of each of the models as well as the main routes of exposure used are discussed in detail below.

Mice. Mice are the most commonly used mammals in scientific research due to the ease of care, the availability of transgenic and disease models, their short life span and their similarities to human beings in terms of genetics and basic physiology. Mouse models of FASD first began to appear in the early 1980s and seminal work by Dr. Kathleen Sulik paved the way for small mammalian models of FASD (47). The route of administration varies from study to study, with the most common models using i.p. injection (47, 113, 156, 157), s.c. injection (111, 158, 159), voluntary drinking paradigms (91, 160, 161), liquid diets (162–164), or oral intubation (165). Most studies employ chronic exposure paradigms (i.e., throughout pregnancy or throughout the third trimester equivalent), but intermittent exposure is also common, particularly in studies where the i.p. route of ethanol administration is used, and where critical periods of vulnerability are being examined (47, 113, 158, 159, 162, 166, 167). The BACs achieved in most studies range between 80–180 mg/dl (for voluntary drinking or liquid diet) and over 200 mg/dl for studies where i.p. injections or oral intubation is used. C57BL/6 is the most common strain of mouse used, but other similar strains are also employed. The ability to genetically manipulate mice can be a huge advantage and many studies into the genetic components associated with FASD have utilized mice as a model (160, 165, 167, 168). A disadvantage with using mice is that the third trimester equivalent of development occurs following birth (see Developmental Timing of Ethanol Exposure). To overcome this, many studies will administer ethanol during the early postnatal period (third trimester equivalent, PND 1–10, see Artificial Rearing), however, issues arise with this method because ethanol exposure occurs outside of the confines of the placental barrier and kinetics and metabolism may be fundamentally different when compared to what happens *in utero*. Despite this, mice are still commonly used, and many common features of FASD that are observed in human subjects are also observed in mice, including craniofacial abnormalities (47, 113, 157), eye malformation (47), growth retardation (162, 163, 166), and cognitive deficits (111, 156, 159–161, 163, 165) [see Ref. (169) for review]. These deficits have been observed across the lifespan (i.e., in neonatal, adolescent, adult, and aged animals) and with all routes of exposure, although the severe growth malformations and facial deficits are often not apparent in models with lower BACs. As well as fundamental studies on the underlying pathologies associated with PNEE, mouse models are also useful for examining potential therapeutics (156).

Rats. Like mice, rats are commonly used as models of FASD. One of the more obvious advantages of rats is their larger size, which makes handing and sampling procedures easier. Rat models also offer an advantage over mouse models because more sophisticated behaviors, including tests of learning and memory and executive function (see Behavioral Manipulations) can be examined more easily in rats, whereas mice have a more limited behavioral repertoire. Like mice, rats have a short lifespan, a gestational period that is analogous to the first two trimesters of human gestation, and neither species requires very sophisticated housing facilities normally (see Developmental Timing of Ethanol Exposure).

Many routes of ethanol administration are used in rat models of FASD: chronic exposure (i.e., throughout gestation) producing moderate stable BACs occurs with liquid diet and voluntary drinking paradigms (55, 69, 87–90, 122–128, 170–180), or if high BACs are preferred oral intubation can be used, either during the gestation period (181), the third trimester equivalent only (56, 95–98, 182–187), or through all three trimester equivalents (78–80). Vapor inhalation (188, 189) is seldom used in current protocols and injection of ethanol i.p. or s.c. does not occur as commonly in rat models and tends to be reserved for mouse models where the effects of ethanol on neuroanatomical features are examined (47, 67, 157).

Like with mice, all the hallmark features of FASD have been demonstrated in rats including growth retardation (174, 188), structural abnormalities (31, 79, 80, 181, 183, 190–193), CNS dysfunction (88, 89, 124, 179, 180, 189, 194), and cognitive deficits (55, 56, 78, 95, 174–178, 184–187). Many of the impairments observed are dose and timing dependent, but are observed across the lifespan and with all routes of ethanol administration. It is also possible to screen potential therapeutics in rat models of FASD and many treatments given either concomitantly with ethanol or following ethanol exposure (i.e., by supplementing offspring after birth) show promise for the mitigation or reversal of some of the cognitive impairments associated with FASD (56, 89, 90, 122).

Guinea pigs. Guinea pig models are utilized in some laboratories as they offer the advantage of being a true *in utero* exposure model because the three trimester equivalents of brain development largely occur during gestation (as opposed to the rat/mouse where the third trimester equivalent is during the early postnatal period). The oral intubation administration route is commonly utilized in guinea pig studies with the dose of ethanol ranging from 3 to 6 g/kg/day (99, 102, 103, 195, 196). In some studies, ethanol administration begins prior to gestation (196) but in the majority of studies ethanol administration begins on GD 1–2 (100, 102, 103, 197, 198). In most studies [excluding (196)], a nutrition/stress control group (which receive sucrose by oral intubation) was included. Results from these studies have indicated that PNEE can cause structural (101, 196–198), functional (103), and cognitive deficits (102, 103, 195) that mimic the human condition. These deficits were observed in neonate (101, 102, 196, 198), adolescent (197, 198), and adult (102, 103, 197, 198) animals. There has been only one study where ethanol administration has been restricted to the third trimester equivalent (classified as GD 43–62) (99) and surprisingly, hippocampal synaptic plasticity and spatial learning were not significantly affected in adult animals even with BACs

of 245 mg/dl (99). Recently, studies utilizing the guinea pig model have been exploring the idea of biomarkers for FASD. Specifically, the accumulation of fatty acid ethyl esters, which form during non-oxidative metabolism of ethanol, in the hair may be a useful indicator of PNEE (199). The advantage of using the guinea pig model for this research is that guinea pigs are the only rodent species that are born with neonatal hair. This line of research may result in the guinea pig model being more widely used in the FASD field. A drawback in using guinea pigs is that the litter size is much smaller than in rats/mice and the longer gestation period can increase the time and costs of a project. Furthermore, guinea pigs may be more difficult to use for behavioral testing as they are not naturally exploratory and may not perform as well as rats in many behavioral tasks (200).

Primates

Because primates are our closest evolutionary ancestors, primate models of FASD are considered a "gold standard." Developmental gestation and length resembles human pregnancy, and more importantly, primates can be used to study more sophisticated behaviors than are possible in rodents or other animal models (201). However, primate research is time consuming (pregnancy length is similar to human beings), expensive, and ethical approval can be difficult to obtain. Because of this, there are very few studies of PNEE that have been conducted in primates and those studies that have been done usually have a very small sample size and there are wide variations in ethanol dosage and administration. For example, one of the first studies conducted by Elton and Wilson (202) allowed four pig-tailed macaques (*Macaca nemestrina*) to consume an orange-flavored ethanol solution prior to conception and throughout pregnancy. While three of the monkeys drank very little of the ethanol and had apparently normal infants, one of the monkeys consumed large amounts of the ethanol throughout her pregnancy and her infant was noted to be hyperactive and tremulous (202). The majority of primate studies utilize the oral intubation method for administering alcohol (104–107), with many studies only giving alcohol once weekly rather than daily, which may more closely resemble human drinking patterns during pregnancy (104, 106, 107). Dosage of ethanol ranges from 0.3 to 5 g/kg and while BACs are not often reported in these studies, in those where they are reported they range from 150 to 250 mg/dl (104, 203). Voluntary drinking paradigms are also used in some studies (0.6 g ethanol/kg/day), and much lower BACs are achieved (20–50 mg/dl) (204–206). There is a large variation in the period of ethanol exposure; in some studies ethanol is administered throughout pregnancy (202, 204, 207), in some it starts after the first month of pregnancy (106) and in others it is intermittent (104, 105, 205). Results from primate studies have shown that ethanol exposure during development produces growth retardation (104–106) as well as behavioral deficits in adolescence and adulthood (104, 105, 204–206) similar to those observed in human beings with FASD.

SUMMARY

There are many different factors to consider when choosing a model to conduct research on FASD. The animal model that is chosen should reflect the specific research question that is to be answered. Depending on what is to be examined, each model offers its own advantages and disadvantages. Peak BAC, developmental timing, route of administration, and stress and nutrition controls should also be considered. Simple invertebrates and vertebrates such as *C. elegans*, *Xenopus*, and zebrafish can be excellent tools for examining the effects of ethanol at a genetic level or on very specific stages of development. Rodents are more commonly used for translational research where the effects of therapeutics can be examined for future use in a clinical population. Non-human primate models are gold standard when it comes to examining complex behavior, but studies are often limited due to small sample sizes, large costs, and time constraints.

BEHAVIORAL MANIPULATIONS

There are many documented behavioral manipulations that have been used to characterize the functional consequences of PNEE in animal models that often correlate with known human dysfunctions [see Ref. (208) for a review of human behavioral work]. With respect to animal models, behavioral experiments are necessary tools when assessing the use of novel therapeutic approaches for PNEE offspring. Here, we will outline five major classes of behavior, including several key behavioral tasks where performance is affected by prenatal ethanol ingestion, injection and inhalation.

MOTOR SKILLS

The cerebellum is a region of the fetal brain that is particularly vulnerable to damage by ethanol *in utero*. Motor hyperactivity is often reported in children with FASD. Children with FASD perform poorly on fine motor coordination and reaching tasks (209) and have deficits in postural balance (210). Recently, children diagnosed with FASD were found to have poor saccade accuracy (211), a task dependent on the cerebellum (212). Motor performance can readily be evaluated in animal models using standardized tasks that include the rotarod, runway, directed reaching, and gait analyses.

In rat pups exposed to ethanol via intubation throughout all stages of pregnancy and during the early postnatal period, the overall volume of the cerebellum and Purkinje cell (PC) numbers were reduced (213). Others have identified the third trimester equivalent as a period of particular vulnerability for PCs (214). PC density is reduced in PND 10 rat offspring exposed to ethanol (gestational intubation) and the ultrastructure of this neural population is modified, indicating a delay in cellular development (215). The widely reported damage to the cerebellum has observable, functional consequences on motor-related behaviors.

Behavioral tasks such as the rotarod, runway tasks, and gait analysis software may be used to examine damage to the cerebellum and related motor structures. In the rotarod task, a rodent is placed in a rotating bar and is required to run on the rod for as long as possible. The rotational speed of the bar can be increased, and the experimenter can then measure the duration of time that the animal can remain on the bar at various speeds (see **Figure 2**). In rodents, it is thought that the motor deficits caused by PNEE are most apparent early in life, and in most cases unseen at adulthood. Bond and DiGiusto (gestational liquid diet) showed these age effects with the anticipated motor hyperactivity in PND 28 and 56 rat offspring, while seeing no evidence of motor impairment at PND 112 (216). Similarly, adult rat offspring

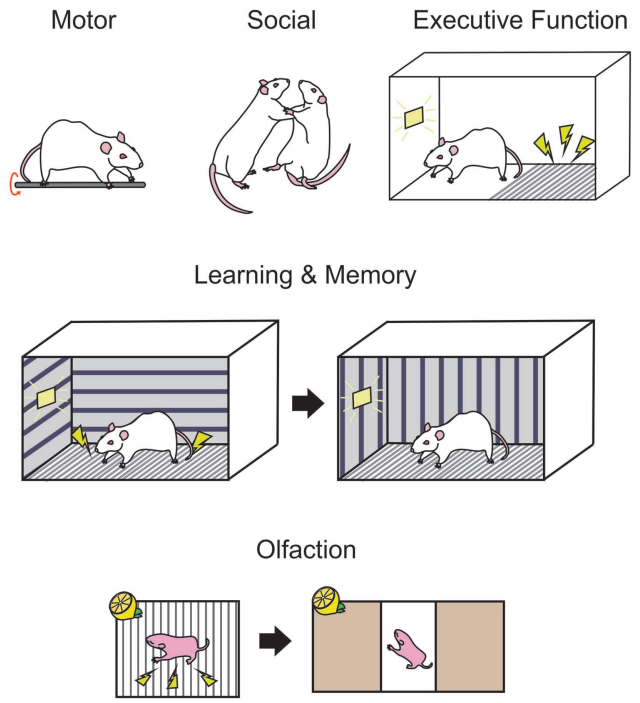

FIGURE 2 | Standardized behavioral measures are used in rodents to examine the functional consequences of developmental ethanol exposure. Motor performance in rodents may be measured on tasks such as the rotarod (upper panel, left-most) where the animal must balance and run on a rotating rod. Social interactions, such as evidence of aggressive behavior can be deduced by observations of wrestling, rearing, and pinning (upper panel, middle) when two conspecifics are paired. Passive avoidance (upper panel, right-most) is a measure of executive behavior in rodents where the animal must learn to inhibit exploratory behavior in order to avoid a shock for the duration of a trial, as indicated by a light. Trace fear conditioning (middle panel) is a hippocampal-dependent behavior where the animal is trained in a context where a signal such as a light indicates a footshock, then after a delay in placed in a novel context and freezing responses can be measured while the light is presented without the footshock. Associative olfactory memories (bottom panel) are formed when an odor, such as lemon is paired with a stimulus such as a footshock and may be tested in a two choice preference chamber, where orientation toward or away from an odor can indicate the presence of a memory for that odor.

(intubation GD 7–20) shows no evidence of motor dysfunction or hyperactivity on a rotarod or open field task (217). During this early window of observation, rats (gestational liquid diet) have been found to be ataxic, exhibiting asymmetrical gait, shorter stride length, and greater step angle than their respective controls (218). Young mice (<PND 60, gestational ethanol in drinking water) perform poorly on the runway and rotarod tasks (219). However, when ethanol administration was restricted to the postnatal period (intubation PND 4–9) as adults (>PND 70), these animals perform poorly on an eyeblink conditioning task, a form of classical conditioning where a light is paired with a puff of air on the eye, causing the animal to blink (220). The impaired performance on this hippocampal-independent task is thought to be due to ethanol-induced damage to the interpositus nucleus of the cerebellum (221). Thomas and colleagues (182) examined the specific timing of postnatal exposure to ethanol (via gastronomy) in relation to cerebellar damage and motor performance. This study identified PND 4/5 as a critical period for ethanol exposure where the greatest deficits could be seen on a parallel bar task at PND 30 and 52, where the width between bars that the animal was required to cross over was gradually widened. This time point also produced the greatest decreases in cerebellar and brainstem weights at PND 55 (182). Others have shown that when ethanol is restricted to the postnatal period (intragastric ethanol PND 2–10), motor hyperactivity in rats persists into adulthood, at least until PND 91 (74).

These studies stress the importance of considering the timing of ethanol administration, the age of the offspring when conducting motor behavior studies, and highlight the need for additional studies in this area in aged animals.

EXECUTIVE FUNCTION

Executive functioning is the ability to use appropriate problem solving in goal-directed behaviors, and includes behaviors such as response inhibition, working memory, and set shifting. These functions have long been thought to be dependent on frontal lobe structures [see Ref. (222) for review] such as the prefrontal cortex, though some argue that extra-frontal-lobe structures may also be involved [see Ref. (223) for review]. In human beings, these behaviors can easily be measured through standardized tests, and they appear to be gravely impacted by prenatal alcohol use [see Ref. (224) for a review]. Children with FASD have difficulties inhibiting responses on the Stroop test (225), a task where an individual must inhibit the natural tendency to read words, being required instead to state the color of the font. In addition, these individuals have difficulty in suppressing saccade responses in visual tasks while waiting for the proper initiating signal (211) and exhibit poor working memory when asked to recall digit spans backwards (226). On the Wisconsin card sorting task, where the subject must detect, use, and change card sorting strategies, individuals with FASD make more errors related to shifting sort strategies (227). In rodents, executive function tasks are complex and a single task often requires the use of response inhibition, working memory, and set shifting among others.

Response inhibition tasks require the subjects to inhibit responses that the organism may be naturally predisposed to perform in particular environments. For example, in rodents, passive avoidance is a task commonly used to show response inhibition. In these tasks, the rodent is placed in a box on a "safe" area, adjacent to a grid floor that will provide a shock if the animal steps onto the grid within a trial. The animal must learn to inhibit the natural tendency to explore new environments and remain on the "safe" side of the test chamber for the entire trial (see **Figure 2** for a schematic). PNEE rats prenatally exposed to ethanol (liquid diet GD 6–16) show impairments in these passive avoidance tasks, at both PND 18 and PND 41–53 (228). In a large rat study, offspring exposed to ethanol (liquid diet GD 5–20) again exhibited impaired passive avoidance of a shock at both PND 17 and PND 48, but not at PND 114, and took longer to spontaneously alter their exploratory strategy on a T-maze where the animal would be confined after visiting a particular arm when tested at PND 16

and PND 63 but not at PND 112 (229). Rats exposed prenatally to ethanol (liquid diet, GD 1–20) have fewer cells in layers II and V of the medial prefrontal cortex, which was correlated with poor performance on a reversal learning task in adulthood [>PND 90; Ref. (230)].

Working memory is a short form of memory where information from a recent experience must be used to perform the appropriate response on a following trial or task. Working memory is a form of memory that is known to primarily require the functional activity of the prefrontal cortex [see Ref. (231) for review of human beings working memory and see Ref. (232) for a review of the cellular mechanisms of working memory], differentiating it from other forms of memory discussed in Section "Learning and Memory." Behavioral tasks that evaluate working memory include delayed matching to sample tasks where a stimulus is provided, followed by a delay then a choice between multiple different stimuli. In these experiments, the organism must remember the initial stimulus then pick the matching stimulus when given a choice after the delay, and is readily adapted for rodent, non-human primates, and human beings. For rodents, the task can be modified to a delayed matching-to-place task in a Morris water maze (MWM), where a platform is located in an arm during a search trial, then after a delay the animal must return to the location of the platform during the search session. When ethanol administration occurs in the third trimester equivalent (gastronomy PND 6–9), these rats perform poorly on the matching-to-place task, at PND 35, PND 105, and PND 180, when the delay between the search and test trials is 2 h (233) though this task requires both intact working and spatial memory.

Set shifting is a complex task that can readily be performed by human beings and non-human primates, with variable evidence from rodents. In rats, set shifting tasks are not as well established as human beings and non-human primate work. In delayed non-matching-to-sample tasks, the subject not only requires functional working memory and inhibitory control but also set shifting where the organism must be able to observe the sample stimulus then shift their attention to choose the non-matching option during the subsequent test trial. In one rat study of delayed non-matching-to-sample, adult PNEE animals (liquid diet GD 1–22) showed no impairments in set shifting (234). Future rodent work in this area may use a unique behavioral task adapted from primate studies (235) in order to fully understand how set shifting may be altered by PNEE in rodents and shed light on the underlying neural substrates for these behaviors. In a study of rhesus monkeys exposed to ethanol (GD 5 – parturition, voluntary drinking), the 32–34 month old offspring had difficulty acquiring a delayed non-matching to sample task (204).

When using animal models to examine the effect of PNEE on executive functions, it is critical to design appropriate tasks for the model in question. Tasks used for one particular species may not be easily applied to other without modifications for the species in question.

LEARNING AND MEMORY

The damaging effects of PNEE on learning and memory have been reliably reported in many species. Here, we will focus on hippocampal-specific learning and memory behaviors in rodents and in human beings. In spatial object memory tasks where a child must remember the location of multiple objects on a semi-random grid, children with FAS were unable to recall objects after a delay and exhibited distorted spatial array when asked to recall where the objects were (236). Additional work with human beings with FASD is necessary to understand the manifestations of neural damage caused by PNEE. Future studies of spatial memory may utilize virtual 3D object-recognition tasks where the subject can undergo PET or fMRI scans while virtually exploring a space (237) as in (238).

In rodents, hippocampal-dependent memory can be assessed in a variety of behavioral tasks including tasks such as the MWM and fear conditioning. PNEE-induced hippocampal damage has been widely reported in rodents (193, 194, 239), for review see Ref. (25, 86).

The MWM is a standard task where a T, plus or open field maze can be submerged in cloudy water. A platform can then be hidden below the surface, and visual detection of the rodent when swimming in the maze. The animal must swim to explore the maze and find the submerged platform to escape the water in multiple training trials where variables such as latency to the platform, swim speed, and distance traveled to platform can be measured. As described above, the MWM can be adapted for many functions, such as delayed matching-to-place (233), which are readily learned by healthy rodents. However, PNEE rodents exhibit significant impairments on this task [rats: liquid diet GD 1–22 (55, 141, 175) and intubation PND 4–9 (56, 95, 184–186); guinea pigs intubation GD 2–67 (102)].

Fear conditioning is a behavioral task that is both easily implemented and readily learned by rodents. Trace fear conditioning occurs when an unconditioned stimulus (US), such as a footshock, follows a conditioned stimulus (CS) such as a tone or a light. Following multiple training sessions, the animal is tested in a novel context similar to the training context and freezing responses are recorded in response to presentation of the CS alone (see **Figure 2**). PNEE rats perform poorly on this task when ethanol is given in the third trimester equivalent [intubation PND 4–9 (240)], with poorest performance observed when ethanol administration occurred from PND 4–6 [intubation (241)].

Other forms of hippocampal memory are impaired by PNEE in rodents. Popovic and colleagues (177) subjected PNEE offspring exposed to gestational ethanol in a liquid diet and/or the early postnatal period to an extensive battery of memory tasks to evaluate performance in spatial learning, orientation, and simple and more complex object recognition. Generally, ethanol-exposed offspring performed poorly, though the impairments in these animals became increasingly evident as the task difficulty increased, with animals treated during the early postnatal period performing worse than others (177).

SOCIAL BEHAVIOR

Social behaviors in human beings and non-humans alike are complex interactions between genetics, early life experiences, and later social learning that can be altered by PNEE [for a review, see Ref. (242, 243)]. For human beings, appropriate behavior in a social context is critical for societal integration, therefore, it is critical to consider that PNEE can shape lifelong behavior, and that FASD

is not simply a childhood disorder as highlighted by Streissguth and colleagues (16) in a longitudinal study examining childhood, adolescents, and adults (16).

Social dysfunctions in human beings with FASD are apparent early in life with altered sleep patterns, increased irritability, and feeding difficulties during infancy (244). Similarly, neonatal rats exposed to ethanol in the early postnatal period (gastronomy PND 2–12) take longer to attach to the nipple and spend less time suckling than controls (245), emit more vocalizations on PND 5 when separated from the dam after pre- and postnatal ethanol exposure [intubation GD 1–22 and PND 2–10 (246)] and are not retrieved by the dam as quickly as unexposed pups [drinking water GD 0–30 (247)]. These negative early life experiences can play a role in shaping social development long term.

For human beings, other social behavioral problems associated with fetal ethanol exposure become apparent at school age. When matched with unexposed children with low verbal IQs, children with FAS have poor coping skills and interpersonal relationship skills according to the Vineland adaptive behavior scale [VABS (248)], performing three standard deviations below the norm for their age. Others have also reported increased aggression in children with FAS (249). In juvenile PNEE rats (liquid diet GD 6–20), the sexually dimorphic play behaviors were reversed where males displayed female behaviors and vice versa (250). Prior to puberty, ethanol-exposed rats (intubation GD 6–19) exhibit more play behavior though males that are more aggressive (see **Figure 2** for a schematic) following puberty than unexposed controls (251). It must be noted, however, the great differences between the complexity of social behavior between human beings and rodents at this age and beyond when drawing parallels between the two species.

Unlike other previously discussed behaviors, disruptions in the social behaviors of adult human beings and rats have been readily shown. In adolescent and adult human beings exposed to ethanol *in utero*, whose average chronological age was 17, the average adaptive functioning as measured by the VABS was equivalent those of a 7-year-old healthy child (16). In this same study, all adolescents and adults were classified in the significant and intermediate categories of the maladaptive behavior section of the VABS including behaviors such as social withdrawal and teasing or bullying of others. In a report on secondary disabilities associated with FASD, Streissguth et al. (252) reported that of adult females exposed to ethanol *in utero*, 40% had drank alcohol while they were pregnant, and over 50% of the children had been removed from the care of the mother. Difficulties in parenting have also been observed in rats that drank ethanol throughout gestation (253). In this study, females exposed to ethanol mother failed to retrieve pups removed from their nests, a task normally accomplished in a short time by control animals. The researchers also observed disorganized and distracted behavior in the mothers. For instance, dams might start carrying a pup part of the way toward the nest, but then drop it and be distracted by self-grooming, eating, or drinking and forget about the retrieval effort (253). Adult males also show disrupted social behaviors at adulthood. Male rat offspring exposed to ethanol prenatally spend less time sniffing other rats at PND 90 than those exposed to maternal saccharin water [gestational ethanol in drinking water; Ref. (254)] and display more aggressive behaviors, including attacks, tail rattling, and chasing in the presence of conspecifics (255). These findings in both rodents and human beings stress that the effects of FASD do not exist in childhood alone, and that they can have effects on the next generation of offspring.

OLFACTION

Olfaction is a complex sense that has recently become of special interest in the area of neurodevelopmental diseases in human beings as early indicators of disease onset, permitting for early intervention [see Ref. (256) for a review]. An early neuropathological report noted significant damage to the olfactory bulbs and stalks in children and fetuses prenatally exposed to alcohol (257) though few studies have examined the functional consequences of this damage. Olfactory abilities can readily be tested in many organisms, from human beings with "Sniffin" sticks (258) to *Drosophila* [see Ref. (259) for a review]. A recent study (260) used two sensory profiling measures filled out by caregivers to examine the sensory abilities of children with FASD. They found that children with FASD have under responsive smell and taste, though the two variables were combined in these forms. In the first study of its kind, children and adolescents exposed to ethanol *in utero* were administered the San Diego Odor Identification Test, where the child is presented with common household odors such as chocolate and peanut butter and must name the odor, revealing significant impairments in the identification of these odors (261). These findings in human beings are compelling, raising questions about the ability for human beings with FASD to discriminate between similar and different odors. Others have reported that fetal ethanol exposure increases infant reactivity to the smell of ethanol after birth, indicating some prenatal sensory memory that persists after birth (262, 263).

Olfaction is the primary sensory modality in rodents and has been extensively studied in the context of memory [for a review see Ref. (264)] and odor identification and discrimination [see Ref. (265) for review]. The olfactory circuitry is susceptible to damage from prenatal ethanol with consistent reports of olfactory bulb damage following various ethanol administration methods in mice [drinking water GD 0–26 (266); injection GD8 (67)] and rats [gastronomy PND 4–9 (267)]. Odor memory can be examined through classical conditioning tasks where an odor can be paired with either an appetitive or aversive stimulus followed by examining the orienting response of the animal to an odor. These tasks can easily be carried out early in life, by pairing an odor with tactile stimulation (268) or a footshock (269) among others (see **Figure 2**). These tasks can be modified for use in juvenile and adult rodents in odor operant boxes, or olfactometers, where the delivery of an odor signals an action for the animal, such as a nosepoke, in order to receive a water reward (270). These olfactometers can also be used to examine odor discriminative abilities in rodents. Odor memory in early life is impaired by prenatal ethanol (liquid diet, GD 6–20) where a PND 3 rat pup is unable to learn aversive (odor + footshock) and appetitive (odor + milk delivery) odor association tasks (271). Interestingly, the impairment in odor associative memory is not apparent at adulthood in an aversive odor association (271). Mice exposed to ethanol *in utero* (drinking water GD 0–26) have poor discriminative abilities when given similar

odors in odor mixture studies though odor associative memory remained intact (266). As with human beings, neonate rats exposed to ethanol *in utero* (liquid diet GD 5–22) where ethanol odor presentation at P15 elicits an altered behavioral response to the odor compared to controls (272).

Disruptions in olfactory memory and odor identification and discrimination as a result of PNEE require more extensive behavioral work to understand how the olfactory circuitry is selectively damaged by ethanol *in utero*. Further behavioral studies in this area are required; though with extensive information available regarding healthy olfactory processing this is a viable area of study for the future.

SUMMARY

The study of the effects of PNEE on offspring has produced extensive evidence of behavioral disruption across multiple neural systems. When describing the damage caused by PNEE, one must consider the interactions between these systems at the behavioral level and therefore make careful choices when designing animal experiments. Together, human beings and animal behavioral impairments can shed light on potential neural targets of or vulnerabilities to PNEE.

CONCLUSION

Fetal alcohol spectrum disorder remains a prevalent problem in our society (7), though there are a great deal of laboratories around the world delineating the mechanisms behind the teratogenic effects of ethanol and the underlying biochemical, molecular, and genetic events that lead to the cognitive deficits characteristic of FASD. Human beings work has identified diagnostic criteria for FASD, which has permitted the proper diagnosis of more individuals that require intervention. Animal models have also been invaluable for this body of work particularly because they allow us to examine different drugs and supplements for their potential therapeutic properties on both neural structures and observable behavior. It is critical for both fields to consider the potential lifelong implications of FASD, as there is a gap in what is understood of PNEE in adults and particularly in aged populations. Moving forward, translational research linking human beings and animal work is imperative in order to paint a vivid picture of damage caused by PNEE and to eventually find a way to overcome some of the devastating effects of PNEE.

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Effects of acute prenatal exposure to ethanol on microRNA expression are ameliorated by social enrichment

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Fetal alcohol spectrum disorders (FASDs) are associated with abnormal social behavior. These behavioral changes may resemble those seen in autism. Rats acutely exposed to ethanol on gestational day 12 show decreased social motivation at postnatal day 42. We previously showed that housing these ethanol-exposed rats with non-exposed controls normalized this deficit. The amygdala is critical for social behavior and regulates it, in part, through connections with the basal ganglia, particularly the ventral striatum. MicroRNAs (miRNAs) are short, hairpin-derived RNAs that repress mRNA expression. Many brain disorders, including FASD, show dysregulation of miRNAs. In this study, we tested if miRNA and mRNA networks are altered in the amygdala and ventral striatum as a consequence of prenatal ethanol exposure and show any evidence of reversal as a result of social enrichment. RNA samples from two different brain regions in 72 male and female adolescent rats were analyzed by RNA-Seq and microarray analysis. Several miRNAs showed significant changes due to prenatal ethanol exposure and/or social enrichment in one or both brain regions. The top predicted gene targets of these miRNAs were mapped and subjected to pathway enrichment analysis. Several miRNA changes caused by ethanol were reversed by social enrichment, including mir-204, mir-299a, miR-384-5p, miR-222-3p, miR-301b-3p, and mir-6239. Moreover, enriched gene networks incorporating the targets of these miRNAs also showed reversal. We also extended our previously published mRNA expression analysis by directly examining all annotated brain-related canonical pathways. The additional pathways that were most strongly affected at the mRNA level included p53, CREB, glutamate, and GABA signaling. Together, our data suggest a number of novel epigenetic mechanisms for social enrichment to reverse the effects of ethanol exposure through widespread influences on gene expression.

Keywords: fetal alcohol syndrome, social behavior, amygdala, ventral striatum, adolescence, gene expression, next generation sequencing

INTRODUCTION

Prenatal ethanol exposure can cause fetal alcohol spectrum disorders (FASDs). With 30% of all women reporting drinking alcohol at some time during pregnancy (1), FASD prevalence in the US and some Western European countries is estimated at 2–5% of school children (2). FASDs are associated with impaired learning and memory, language development, and abnormal social behavior [reviewed in Ref. (3)]. The social behavior changes seen in adolescents can resemble those that are typically associated with autism.

Behavioral deficits can also be seen in animal models of prenatal ethanol exposure. Acute exposure on gestational day 12 (G12) in rats leads to decreased social investigation and play fighting, as well decreased social motivation in late adolescence and adulthood (4, 5). To date, amelioration of social behavior deficits from prenatal ethanol exposure has largely focused on behavioral interventions. However, social experience with typically developing peers has been found to be important for improving social skills and increasing social interaction in autistic children. In previous work, we showed that a form of social enrichment (housing ethanol-exposed rats with non-exposed control rats) could normalize the social motivation deficit phenotype seen in both males and females at postnatal day 42 (P42) following gestational ethanol exposure at G12 (5).

The amygdala is critical for normal social behavior. Lesions of the amygdala alter social functions in human beings and experimental animals (6), and developmental changes in the amygdala have been described in autism (7). The amygdala is thought to regulate social behavior in part through connections with the prefrontal cortex, thalamus, and basal ganglia (8, 9). Within the basal ganglia, the ventral striatum has been viewed as a critical integration center for social/emotional signals from the amygdala, as well as spatial/contextual information from the hippocampus, reward/motivational signals from midbrain dopamine neurons, and cognitive signals from the prefrontal cortex (9).

At the cellular level, the amygdala is composed of a group of 13 sub-nuclei located in the medial temporal lobe (8). These nuclei may be divided into four subdivisions (10): (1) basolateral (which includes the lateral, basolateral, and basomedial nuclei), (2)

cortical like (including nucleus of the lateral olfactory tract, bed nucleus of the accessory olfactory tract, the cortical nucleus, and the periamygdaloid cortex), (3) centromedial (central and medial nuclei, and the amygdaloid part of the bed nucleus of stria terminalis), and (4) other (which includes anterior amygdala area, the amygdalo–hippocampal area, and the intercalated nuclei). Developmentally, many amygdala nuclei derive from the medial ganglionic eminence (i.e., are diencephalic) (11), although the cortical amygdaloid nuclei are telencephalic in origin (12). Neuronal types differ considerably among the subdivisions of the amygdala (10). In the basolateral group, approximately 70% of neurons are thought to be glutamatergic (pyramidal, spiny, or class I neurons). This division also contains interneurons such as GABAergic nonspiny stellate cells of the cortex (called S cells, stellate, or class II neurons). In contrast, within the central nucleus, the majority of cells are thought to be GABAergic.

microRNAs (miRNAs) are a class of short, hairpin-derived RNAs that repress gene expression at the post-transcriptional level. Mature miRNAs of ~20 nt in length canonically bind to complementary sequences found in the 3′ untranslated region of messenger RNAs (mRNAs), thereby repressing translation by ribosomes. In neurons, miRNAs also play a role in compartmentalizing specific mRNA translation in subcellular components, including axons (13) and synapses [reviewed in Ref. (14)]. Dysregulation of miRNAs have recently been associated with a variety of neurodegenerative diseases as well as alcohol consumption in human beings (15) and rodent fetal exposure models [reviewed in Ref. (16)].

In this study, we extend our previous characterization (5) of selected alterations in gene expression in the amygdala and ventral striatum as a consequence of prenatal ethanol exposure and an environmental manipulation (social enrichment) in rats. Using the same tissue samples used in our previous study (5), we analyzed miRNA from the amygdala and ventral striatum of 72 adolescent male and female rats. Samples were pooled to 24 for each brain region and analyzed by RNA-Seq and Affymetrix miRNA arrays. We identified many miRNAs with nominally significant changes due to prenatal ethanol exposure or social enrichment. Some of the gene expression changes due to ethanol were reversed by social enrichment. Pathway enrichment analysis was also performed on the top changed miRNAs. We comprehensively integrate these findings with our existing mRNA data to determine whether the target mRNAs of the altered miRNAs showed evidence of changing, using whole transcriptome microarray data from the same rats. Further, we broaden our mRNA analysis by considering all possible genes in the context of canonical pathways related to brain function. This additional analysis highlights striking reversals following social enrichment in p53, CREB, glutamate, and GABA signaling. Altogether, these analyses suggest possible mechanisms for social enrichment to reverse some of the effects of prenatal ethanol exposure.

MATERIALS AND METHODS

ANIMALS

Treatment of animals, as well as behavioral and mRNA expression outcomes, were described in Middleton et al. (5). Briefly, timed pregnant Long Evans rats (Harlan, Indianapolis, IN, USA) were received on G4, with G1 designated as the first day on which a sperm-positive plug was noted. These rats were housed at the Department of Veterans Affairs Medical Center (VAMC) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in Syracuse, NY. All procedures were approved by the Institutional Animal Care and Use Committees at both the Syracuse VAMC and SUNY Upstate Medical University, and were in accordance with the guidelines for animal care established by the National Institutes of Health. Rooms were maintained on a reverse 12-h light/dark cycle at 22°C (lights off at 7:00 a.m.).

Animals were exposed to ethanol prenatally as described previously (5). On G12, dams received an initial intraperitoneal (i.p.) injection of ethanol (2.9 g/kg as a 20% v/v solution in physiological saline) followed by a second i.p. injection 2 h later of 1.45 g/kg ethanol (**Figure 1**). Control animals received i.p. injections equivalent volumes of saline at the same timepoints. This method of ethanol administration leads to blood ethanol concentrations of 287 ± 3.5 mg/dl within 15 min of the second injection. After birth, all litters were culled to 10 pups within 24 h, with equal ratios of males/females as best as possible. On P21, litters were weaned and male and female offspring were housed separately. After social behavior testing (described below), animals were injected intraperitoneally with 100 mg/kg ketamine and 10 mg/kg xylazine prior to decapitation. Brains were rapidly removed, snap-frozen on dry ice, and stored at –80°C until used for RNA extraction (see below).

Our previous behavioral study on the same cohort of rats, described in Middleton et al. (5), examined the effects of a form of environmental manipulation termed social enrichment, during the post-weanling and early adolescent period (P21–P42) in animals prenatally exposed to ethanol (**Figure 1**). This treatment involved housing experimental animals (offspring of saline- or ethanol-injected dams) with either 2 or 3 same-sex littermates (non-enriched condition) or 2 or 3 novel same-age, same-sex animals from a non-treated dam (social enrichment condition). The effect of this manipulation on social behavior was evaluated on P42 by testing their responses to the introduction of another same-age, same-sex rat (from an untreated dam) over the course of a 10-min social interaction test (SIT). Among the behavioral measures assessed during the SIT were social investigation (sniffing of the novel rat's body), contact behavior (grooming, crawling over or under the novel rat), play fighting (following, chasing, nape attacks, pinning), and social motivation (a coefficient of social preference vs avoidance of the novel rat). The most significant finding from our prior analysis was the finding that prenatal ethanol exposure negatively affected social motivation performance in both male and female rats following prenatal ethanol exposure, but this impairment was completely reversed by social enrichment.

MOLECULAR PROFILING

The present study was designed to determine the potential molecular substrates of decreased social motivation following prenatal ethanol exposure and its reversal by social enrichment. We first dissected the whole amygdala and ventral striatum from a total of 72 42-day-old male and female rats, using established anatomical landmarks, as described previously (5). After isolating the regions

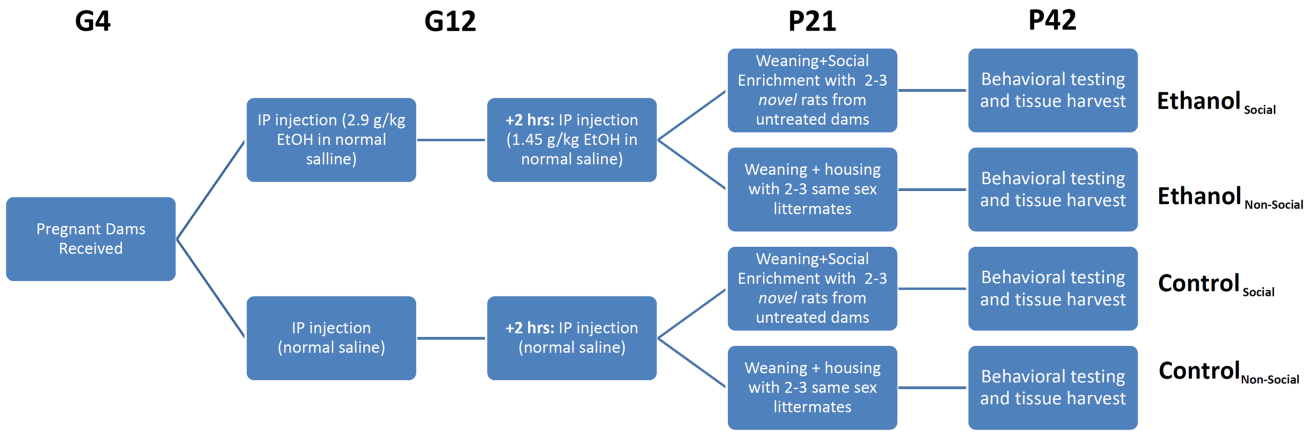

FIGURE 1 | Experimental overview. Timed pregnant female rats were received on gestational day 4 (G4) and split into two treatment groups – ethanol (EtOH) or saline. Each animal was injected twice on G12 with EtOH or an equivalent volume of saline. Pups were delivered in their home cage, litters culled to 10 pups with a 1:1 ratio of males:females, and remained in their home cage until weaning. On weaning, rats were separated into non-social and social-enriched conditions for 3 weeks (P21-42), during which they were housed with 2–3 age- and sex-matched rats from their own litter or a novel litter from an untreated dam, respectively. All animals underwent behavioral testing using the social interaction test (SIT), which measures social investigation, contact behavior, play fighting, and social motivation (social preference vs avoidance for the novel rat). After testing, rats were euthanized and tissue harvested for RNA profiling.

of interest, total RNA was purified using the RNeasy kit (Qiagen, Valencia, CA, USA). RNA yield and quality were assessed by UV spectrophotometry and the Agilent Technologies Bioanalyzer. A total of 144 RNA samples were purified from the 72 rats, including 9 males and 9 females for each of the 4 treatment groups: (1) ethanol-exposed/non-enriched, (2) ethanol-exposed/socially enriched, (3) saline-exposed/non-enriched, and (4) saline-exposed/socially enriched. For all subsequent molecular assays described in this report, three pools of RNA were created for each brain region using equal amounts of RNA from the nine male or nine female rats within each treatment group. Thus, a total of 24 RNA samples from each brain region were examined (12 male pools, 12 female pools), representing a total of 6 per treatment condition. We point out that this pooling strategy preserved our ability to look at the contribution of different brain regions and genders on expression level, and was made purely to reduce cost.

High-resolution quantification of miRNA expression was performed using small RNA-sequencing from 1 μg of each pool of total RNA according to the TruSeq Small RNA Sample Prep kit (Illumina, San Diego, CA, USA). Subsequent purification methods including gel purification of small RNAs 20–30 nt in length, resulted in more than 90% of all reads in the sample attributed to miRNAs. Libraries were indexed and multiplexed in sets of 8 (6 sets total) prior to sequencing (single-end, 37 cycles) using Reagent Kit v3 reagents on a MiSeq Benchtop Sequencer (Illumina, San Diego, CA, USA). Raw sequence FASTQ files were imported into Partek Flow software for initial analysis. Base calls below a phred score of 20 were trimmed from the reads. These were then aligned to the Rn5 version of the rat genome using the Bowtie algorithm (17). The aligned reads were then quantified against the miRBase 21 transcript annotations for both precursor and mature miRNAs (18). Reads from miRNA genes were normalized and scaled to reads per million (RPM) for comparison between samples and comparison with the microarray data in Partek Genomics Suite.

In order to validate the changes seen by RNA-Seq, we also examined the samples using the GeneChip miRNA 2.0 array (Affymetrix, Santa Clara, CA, USA). Samples from the same pooled RNA were prepared using the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA, USA). Arrays were hybridized, washed, stained, and scanned according to manufacturer protocol and the data exported and normalized using RMA in Partek Genomics Suite.

We also examined the relationship of the miRNA data to our previously described mRNA dataset generated from the same pooled rat brain RNA samples; see Ref. (5). Those data were generated with the Rat ST Gene 1.0 array (Affymetrix, Santa Clara, CA, USA), normalized using RMA and imported into Partek Genomics Suite for analysis alongside the miRNA microarray and RNA-Seq data. We point out that while our previous report focused only on a subset of 663 mRNAs related to 17 gene ontology terms of interest (social, anxiety, fear, autism, synapse, synaptic, norepinephrine, neuropeptide, cannabinoid, opioid, oxytocin, GABA, glutamate, glycine, serotonin, dopamine, neurotransmitter), the present study examined the potential miRNA modulation of all predicted target mRNAs in the data set as well as the potential enrichment of modulated miRNAs and mRNAs within curated, canonical pathways using the QIAGEN Ingenuity® Pathway Analysis (IPA) software.

All of the raw and normalized microarray and RNA-Seq expression data generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO accession # GSE60901, which includes microarray subseries GSE60819 and RNA-Seq subseries GSE60900).

MOLECULAR SUBSTRATE ANALYSIS

The major finding from our previous behavioral study was that social motivation was significantly decreased in male and female rats prenatally exposed to ethanol at G12, and that this was reversed by social enrichment. The focus of the present study was to identify

the molecular substrates underlying the social motivation deficit and its reversal. To accomplish this, our primary analysis utilized a 3 way ANOVA (2 genders × 2 prenatal diets × 2 postnatal treatments) for each brain region to identify miRNAs with highly consistent changes (1-tailed $p < 0.1$ for both RNA-Seq and microarray analyses) due to prenatal ethanol exposure and social enrichment. These ANOVAs were followed by Fisher's *post hoc* testing to compare specific groups within each brain region. Notably, after identifying the miRNAs with the most robust main effects within each brain region, we also performed exploratory four-way ANOVAs, using the previous three factors plus brain region, and examined the top miRNAs for any evidence of significant interactions (e.g., brain region × diet, brain region × gender, brain region × social enrichment, and all other combinations of interactions). The results of this exploratory analysis are provided as Supplementary Material.

The top findings from the analysis of individual miRNAs were displayed in table format (**Tables 1** and **2**). We include in these results, individual miRNAs that were significantly changed ($p < 0.05$) according to the RNA-Seq analyses, but were not probed by the Affymetrix GeneChip miRNA 2.0 array.

A combined analysis of miRNA data and mRNA data was then performed. The mRNA targets of the most robustly affected miRNAs were mapped using the miRNA target filter workflow of QIAGEN Ingenuity® Pathway Analysis (IPA) software. We also examined the entire mRNA dataset for specific canonical neuronal pathway effects using IPA software, using a threshold of $p < 0.1$ from the ANOVA *post hoc* testing.

We note that 0.1 was chosen as the *de facto* threshold for significance throughout most of our analyses because of the combined use of multiple platforms for cross-validation or the combined use of multiple genes within networks as well as miRNAs and their target mRNAs.

RESULTS

microRNAs ARE ALTERED BY PRENATAL ETHANOL EXPOSURE AND SOCIAL ENRICHMENT

We performed a global screen of all known, curated miRNA molecules. To ensure full coverage, a conservative cross-platform approach employing both miRNA microarrays and RNA-Seq was used for identification of potential miRNA of interest. Quantification of miRNAs was based primarily on small RNA-Sequencing, which has increasingly emerged as the gold standard of miRNA quantification technologies, owing to its greater sensitivity and dynamic range compared to other techniques. Orthogonal validation was performed using Affymetrix miRNA GeneChips. The application of these two complementary technologies improved our capacity to discover relevant miRNAs that may have been overlooked had a single quantification method been employed. On the other hand, because miRNA microarrays are limited to the interrogated content of the arrays at the time of manufacture, we also included in our analyses those miRNAs that were found only by small RNA-Seq.

The Affymetrix GeneChip miRNA 2.0 array that we used included probes for 780 *Rattus norvegicus* precursor and mature miRNAs (representing approximately half that number of unique miRNAs). The RNA-Seq analysis that we employed identified 1063 precursor and mature miRNAs listed in the miRBase 21 annotation (18). A total of 601 miRNAs could be cross-referenced based on exact sequence conservation of the array probe and RNA-Seq annotation. In the space that follows, we describe first the changes due to fetal ethanol or postnatal social enrichment in these miRNAs, as seen in the amygdala and/or ventral striatum of both genders of rats.

Ethanol effects

In the amygdala, out of the 601 total miRNAs we identified a total of 291 miRNAs with consistent changes (in the same direction) due to ethanol in non-enriched animals representing 48% directional concordance. Of these, 12 miRNAs were changed in both platforms (at the $p < 0.1$ level) (**Table 1**, upper). An additional 17 miRNAs only found by RNA-Seq were also observed to change (at $p < 0.05$ level) due to ethanol effects in non-enriched rats (**Table 1**, upper). In rats subjected to social enrichment, we observed a total of 275 (46%) directionally concordant changes, with 1 miRNA changed ($p < 0.1$) in both platforms and 10 additional miRNAs significantly changed ($p < 0.05$) that were only found by RNA-Seq (**Table 1**, upper).

In the ventral striatum, 281 of the miRNAs (47%) showed concordant directional changes due to ethanol in non-enriched animals, with 3 changed (at the $p < 0.1$ level) in both platforms and 11 additional miRNAs significantly changed that were only found by RNA-Seq (**Table 2**, upper). In rats subjected to social enrichment, a total of 284 (47%) miRNAs showed directionally concordant changes, with 3 changed ($p < 0.1$) in both platforms, and 13 additional miRNAs significantly changed ($p < 0.05$) that were only found by RNA-Seq (**Table 2**, upper).

Social enrichment effects

For social enrichment effects in the amygdala of control rats, 251 (42%) miRNAs showed directional concordance with 9 miRNAs changed in both platforms at the $p < 0.1$ level and 11 additional miRNAs found by RNA-Seq (**Table 1**, lower). In corresponding ethanol-exposed rats, 286 (48%) showed directional concordance with 7 miRNAs changed in both platforms at the $p < 0.1$ level and 12 additional miRNAs found by RNA-Seq (**Table 1**, lower).

In the ventral striatum of control rats, 267 (44%) showed concordant directional changes due to enrichment, with 11 changed (at the $p < 0.1$ level) in both platforms and 9 additional miRNAs changed (at the $p < 0.05$ level) by RNA-Seq (**Table 2**, lower). In corresponding ethanol-exposed rats, 273 (45%) showed directional concordance with 7 miRNAs changed in both platforms at the $p < 0.1$ level and 13 additional miRNAs found by RNA-Seq (**Table 2**, lower).

We note that most of the 53 concordant miRNA differences found by both miRNA and RNA-Seq were similar in magnitude. However, the magnitude of the difference found by RNA-Seq exceeded the difference found by microarray by at least 50% for 10 miRNAs, while the difference by array was 50% greater than RNA-Seq for only 2 miRNAs. These observations lend additional support for the growing awareness that RNA-Seq appears to have greater dynamic range than microarray-based expression profiling.

Table 1 | Nominally significant miRNAs in amygdala.

miRNA	miRBase 21 accession	RNA-Seq		Microarray	
		Fold change	*p*-Value	Fold change	*p*-Value
ETHANOL EFFECT IN NON-ENRICHED RATS [$ETHANOL_{NON\text{-}SOCIAL}$ VS $CONTROL_{NON\text{-}SOCIAL}$]					
miR-1843a-3p	MIMAT0024848	−1.50	0.097	−1.62	0.045
miR-221-5p	MIMAT0017163	−1.38	0.031	−1.15	0.004
miR-29c-3p	MIMAT0000803	−1.18	0.038	−1.12	0.068
miR-384-5p	MIMAT0005309	−1.12	0.063	−1.21	0.002
miR-412-3p	MIMAT0003124	−2.57	0.025	−1.20	0.032
mir-129-1	MI0000902	−1.31	0.079	−1.27	0.089
mir-138-2	MI0000911	−1.20	0.065	−1.28	0.014
mir-155	MI0025509	1.34	0.057	1.24	0.026
mir-322-2	MI0031763	−1.23	0.064	−1.36	0.017
mir-34c	MI0000876	2.83	0.072	1.52	0.013
mir-496	MI0012622	−1.54	0.010	−1.18	0.031
mir-9a-2	MI0000840	−1.17	0.062	−1.26	0.045
miR-148a-5p	MIMAT0035724	−1.19	0.011		
miR-15b-3p	MIMAT0017093	4.50	0.024		
miR-221-3p	MIMAT0000890	−1.27	0.010		
miR-222-3p	MIMAT0000891	−1.23	0.030		
miR-299a-5p	MIMAT0000901	−1.55	0.049		
miR-301b-3p	MIMAT0005304	2.85	0.017		
miR-448-3p	MIMAT0001534	4.07	0.040		
miR-449a-5p	MIMAT0001543	3.84	0.044		
miR-495	MIMAT0005320	−1.46	0.035		
miR-6329	MIMAT0025068	−2.38	0.003		
miR-667-3p	MIMAT0012852	−1.30	0.017		
mir-204	MI0000946	3.97	0.042		
mir-299a	MI0000970	−1.46	0.007		
mir-3084a	MI0030358	3.69	0.034		
mir-3556b-2	MI0031769	−1.18	0.006		
mir-448	MI0001639	3.59	0.050		
mir-6329	MI0021853	−1.45	0.045		
ETHANOL EFFECT IN SOCIALLY ENRICHED RATS [$ETHANOL_{SOCIAL}$ VS $CONTROL_{SOCIAL}$]					
mir-218a-2	MI0000957	1.33	0.054	1.22	0.029
let-7c-2	MI0000831	1.38	0.018		
let-7c-5p	MIMAT0000776	1.39	0.005		
miR-148a-5p	MIMAT0035724	−1.17	0.022		
miR-195-5p	MIMAT0000870	1.25	0.039		
miR-6319	MIMAT0025056	−11.33	0.002		
miR-6324	MIMAT0025063	−25.25	0.005		
miR-872-3p	MIMAT0005283	−1.39	0.002		
mir-195	MI0000939	1.26	0.044		
mir-6324	MI0021848	−4.38	0.008		
mir-708	MI0006160	1.25	0.030		
SOCIAL ENRICHMENT EFFECT IN CONTROL RATS [$SOCIAL_{CONTROL}$ VS $NON\text{-}SOCIAL_{CONTROL}$]					
miR-106b-5p	MIMAT0000825	−1.35	0.001	−1.39	0.008
miR-218a-5p	MIMAT0000888	−1.30	0.032	−2.15	0.010
miR-30c-5p	MIMAT0000804	−1.09	0.096	−1.39	0.002
miR-674-3p	MIMAT0005330	−1.29	0.024	−1.50	0.072
miR-96-5p	MIMAT0000818	−3.78	0.099	−1.32	0.001
miR-9a-3p	MIMAT0004708	−1.11	0.087	−1.51	1E-06
mir-218b	MI0015428	−1.31	0.036	−1.42	0.029

(Continued)

Table 1 | Continued

miRNA	miRBase 21 accession	RNA-Seq		Microarray	
		Fold change	*p*-Value	Fold change	*p*-Value
mir-503-2	MI0031773	−2.42	0.062	−1.46	0.001
mir-544	MI0012593	5.25	0.022	1.24	0.068
miR-106b-5p	MIMAT0000825	−1.35	0.001	−1.39	0.008
miR-218a-2-3p	MIMAT0004740	11.03	0.045		
miR-221-3p	MIMAT0000890	−1.20	0.034		
miR-299a-5p	MIMAT0000901	−1.57	0.044		
miR-3084d	MIMAT0035745	−1.84	0.041		
miR-503-3p	MIMAT0017224	−1.76	0.032		
miR-6319	MIMAT0025056	2.16	0.043		
miR-6329	MIMAT0025068	−1.58	0.042		
miR-667-3p	MIMAT0012852	−1.34	0.009		
miR-872-3p	MIMAT0005283	1.28	0.011		
mir-6319-1	MI0021841	4.26	0.028		
mir-708	MI0006160	−1.24	0.033		
SOCIAL ENRICHMENT EFFECT IN ETHANOL RATS [$SOCIAL_{ETHANOL}$ VS NON-$SOCIAL_{ETHANOL}$]					
miR-17-1-3p	MIMAT0004710	1.75	0.058	1.21	0.008
miR-204-5p	MIMAT0000877	−2.83	0.084	−3.52	0.057
miR-376b-5p	MIMAT0003195	1.36	0.015	2.29	9.7E-07
miR-378a-5p	MIMAT0003378	−1.98	0.014	−1.38	0.002
miR-384-5p	MIMAT0005309	1.12	0.060	1.12	0.038
miR-874-5p	MIMAT0017290	−1.91	0.047	−1.28	0.041
mir-19a	MI0000849	−1.88	0.079	−1.25	0.085
miR-142-3p	MIMAT0000848	−2.53	0.013		
miR-183-5p	MIMAT0000860	−358.64	0.049		
miR-199a-3p	MIMAT0004738	−1.51	0.027		
miR-222-3p	MIMAT0000891	1.21	0.049		
miR-301b-3p	MIMAT0005304	−2.21	0.041		
miR-3068-5p	MIMAT0024845	1.47	0.013		
miR-3557-3p	MIMAT0017820	−1.98	0.014		
miR-379-3p	MIMAT0004791	1.31	0.019		
miR-493-3p	MIMAT0003191	−2.80	0.050		
miR-6329	MIMAT0025068	1.84	0.049		
mir-296	MI0000967	1.45	0.034		
mir-299a	MI0000970	1.41	0.015		

Comparisons and fold changes measured using RNA-Seq and, where available, validated by Microarray quantification. Table contains miRNAs with consistent changes at an uncorrected p-value cutoff of 0.1 or less for both RNA-Seq and Microarray data sets, or miRNAs that were not present on the array but were observed to change in the RNA-Seq data with an uncorrected p-value cutoff of 0.05 or less.

From this point forward, we specifically chose to further examine the ethanol effect in non-enriched rats [N (EvC)] and the social enrichment effect in ethanol-exposed rats [E (SvN)], as these groups exhibited a striking reversal in social motivation (5). Hierarchical cluster analysis showed distinct expression patterns in groups of miRNAs, including some with directional reversals resulting from social enrichment (**Figure 2**). Except for one (miR-381-5p), none of the miRNAs in this subset showed any main effects of gender (Datasheet S1 in Supplementary Material). Thus, they do not appear to have gender-specific gene effects.

This comparative analysis also revealed several notable individual miRNAs (**Table 3**). First, miR-874-5p was decreased in both the amygdala and ventral striatum. On the other hand, mir-183 was affected by social enrichment in both brain regions, with its mature miR-183-5p showing a striking 300-fold decrease in the amygdala and 5-fold increase in the ventral striatum. Thus, brain region clearly influenced the miRNA results.

In addition to single miRNAs, we also examined whether our miRNAs of interest belonged to the same miRBase families, which are clusters of highly homologous sequences. Interestingly, the miRNA families that had common members, including mir-122 (miRBase family accession # MIPF0000095) and mir-8 (miRBase family accession # MIPF0000019) had very similar fold changes and *p*-values within the same comparisons. We note that the identical results between members of the same miRNA family likely reflect the fact that our RNA-Seq analysis could not distinguish

Table 2 | Nominally significant miRNAs in ventral striatum.

miRNA	miRBase 21 accession	RNA-Seq		Microarray	
		Fold change	*p*-Value	Fold change	*p*-Value
ETHANOL EFFECT IN NON-ENRICHED RATS [ETHANOL$_{NON-SOCIAL}$ VS CONTROL$_{NON-SOCIAL}$]					
let-7c-1	MI0000830	1.17	0.035	1.07	0.009
let-7c-2-3p	MIMAT0017088	1.91	0.041	1.17	0.084
mir-542-1	MI0003528	1.36	0.090	1.21	0.003
miR-1247-5p	MIMAT0035721	−1.79	0.047		
miR-133b-3p	MIMAT0003126	11.64	0.016		
miR-345-3p	MIMAT0004655	1.68	0.012		
miR-489-5p	MIMAT0017196	−2.00	0.032		
miR-493-3p	MIMAT0003191	−2.12	0.035		
miR-540-5p	MIMAT0017211	−1.31	0.030		
miR-6314	MIMAT0025047	2.35	0.047		
mir-122	MI0000891	−5.54	0.004		
mir-1306	MI0021537	−1.48	0.046		
mir-3591	MI0015471	−5.54	0.004		
mir-6314	MI0021832	2.36	0.044		
ETHANOL EFFECT IN SOCIALLY ENRICHED RATS [ETHANOL$_{SOCIAL}$ VS CONTROL$_{SOCIAL}$]					
miR-200b-3p	MIMAT0000875	4.55	0.065	3.94	0.053
miR-26b-3p	MIMAT0004714	1.55	0.041	1.08	0.012
mir-542-2	MI0031781	−1.34	0.037	−1.22	0.052
miR-133b-3p	MIMAT0003126	3.18	0.011		
miR-200a-3p	MIMAT0000874	6.48	0.044		
miR-344g	MIMAT0025052	−3.55	0.044		
miR-3553	MIMAT0017814	5.51	0.023		
miR-493-3p	MIMAT0003191	−2.03	0.026		
miR-532-3p	MIMAT0005323	−1.61	0.041		
miR-540-5p	MIMAT0017211	−1.27	0.023		
miR-582-5p	MIMAT0012833	−1.41	0.045		
mir-183	MI0000928	5.47	0.022		
mir-200a	MI0000943	6.74	0.048		
mir-3548	MI0015404	6.74	0.048		
mir-3553	MI0015410	5.47	0.022		
mir-3577	MI0015449	4.48	0.007		
SOCIAL ENRICHMENT EFFECT IN CONTROL RATS [SOCIAL$_{CONTROL}$ VS NON-SOCIAL$_{CONTROL}$]					
miR-155-5p	MIMAT0030409	1.61	0.098	1.47	2E-04
miR-24-2-5p	MIMAT0005441	1.14	0.085	1.28	0.031
miR-27a-5p	MIMAT0004715	3.55	0.038	1.31	2E-04
miR-299a-3p	MIMAT0017167	−1.54	0.018	−1.86	0.032
miR-434-5p	MIMAT0017307	1.11	0.030	1.45	1E-04
miR-487b-3p	MIMAT0003200	−1.15	0.037	−1.46	0.003
mir-1247	MI0030348	−1.81	0.041	−1.13	0.071
mir-31b	MI0015412	1.27	0.079	1.16	0.042
mir-487b	MI0003547	−1.14	0.033	−1.22	0.042
mir-653	MI0012600	−1.78	0.058	−1.41	0.011
mir-673	MI0006158	1.19	0.068	1.18	0.075
miR-1247-5p	MIMAT0035721	−1.80	0.045		
miR-145-5p	MIMAT0000851	1.49	0.017		
miR-224-5p	MIMAT0003119	11.41	0.019		
miR-3592	MIMAT0017895	1.63	0.005		
miR-3594-3p	MIMAT0017899	3.43	0.035		

(Continued)

Table 2 | Continued

miRNA	miRBase 21 accession	RNA-Seq		Microarray	
		Fold change	*p*-Value	Fold change	*p*-Value
miR-382-3p	MIMAT0003202	1.63	0.005		
miR-653-5p	MIMAT0012838	−1.84	0.038		
miR-98-5p	MIMAT0000819	−1.10	0.047		
mir-3577	MI0015449	−4.40	0.008		
SOCIAL ENRICHMENT EFFECT IN ETHANOL RATS [$SOCIAL_{ETHANOL}$ VS NON-$SOCIAL_{ETHANOL}$]					
miR-141-3p	MIMAT0000846	3.60	0.010	1.46	0.065
miR-182	MIMAT0005300	3.51	0.001	1.27	0.050
miR-200b-3p	MIMAT0000875	5.09	0.020	5.45	0.058
miR-320-3p	MIMAT0000903	−1.20	0.055	−1.27	0.093
miR-323-5p	MIMAT0004637	1.23	0.023	1.17	0.071
miR-874-5p	MIMAT0017290	−1.88	0.001	−1.60	0.099
mir-148a	MI0030350	−1.20	0.090	−1.21	0.076
miR-188-5p	MIMAT0005301	−4.04	0.019		
miR-200a-3p	MIMAT0000874	7.03	0.041		
miR-344g	MIMAT0025052	−3.64	0.038		
miR-3553	MIMAT0017814	4.55	0.029		
miR-381-5p	MIMAT0017220	3.47	0.047		
miR-532-3p	MIMAT0005323	−1.64	0.033		
miR-6318	MIMAT0025055	2.13	0.008		
mir-183	MI0000928	4.70	0.026		
mir-200a	MI0000943	6.81	0.048		
mir-3084a	MI0030358	2.82	0.032		
mir-344g	MI0021837	−1.88	0.012		
mir-3548	MI0015404	6.81	0.048		
mir-3553	MI0015410	4.70	0.026		

Comparisons and fold changes measured using RNA-Seq and, where available, Microarray quantification. Conventions same as ***Table 1****.*

the two isoforms using the standard read count quantification algorithm that we employed. Additional experiments on precursor forms of these miRNAs would be needed to elucidate the effects of individual miRNAs within such families. This would be particularly interesting for the mir-8 family, whose members have been implicated in synaptic development (19).

Most importantly, we also noted several miRNAs that appeared to significantly reverse their expression levels in the amygdala after social enrichment in ethanol-exposed animals. These include precursor miRNAs mir-204 and mir-299a as well as mature miRNAs miR384-5p, miR-222-3p, and miR-301b-3p. Because these molecular changes parallel the behavioral changes, it is possible that they may be more directly related to the primary mechanisms underlying each phenotypic effect.

DIFFERENTIAL MODULATION OF TARGETED MESSENGER RNAs CONVERGE ON CELL SIGNALING AND MORPHOLOGY

In order to elucidate the large-scale functional changes being affected by ethanol and social enrichment, we performed functional network enrichment analysis using QIAGEN Ingenuity® IPA software. The IPA core analysis workflow determines key gene regulatory networks that are overrepresented in any given set of molecules. Additionally, the activation or inhibition of upstream and downstream molecules can be predicted based on existing data and overlaid on any network to show how the overall network is affected.

We first performed this analysis using only the specific miRNAs validated using both RNA-Seq and array that were altered by ethanol in non-social animals and by social enrichment in ethanol-exposed animals (**Tables 1** and **2**). The combined networks obtained by a core analysis of these data were merged and examined using data from the amygdala and ventral striatum. Overall, the functions represented by the resulting merged network included several cell signaling molecules. Hereafter, we refer to this merged network as a Cell Signaling network (**Figure 3**). Key hub molecules in this network of miRNAs and target mRNAs include p53, IGF1R, TNF, and several others. Most interestingly, the molecule activity predictor tool in IPA generally suggested a large-scale activation (orange colors) of this network in the amygdala and inhibition (blue colors) in the ventral striatum.

The miRNA data were also integrated with mRNA data ($p < 0.1$) derived from the same tissues (5) using the IPA miRNA target filter workflow. Relationships included experimentally determined data from the Ingenuity® curated database and highly predicted targets from the target prediction databases in TargetScan (20), miRecords (21), and TarBase (22). We note that the miRNA-target predictions are based on sequence complementarity between the miRNA seed sequence and the target mRNA,

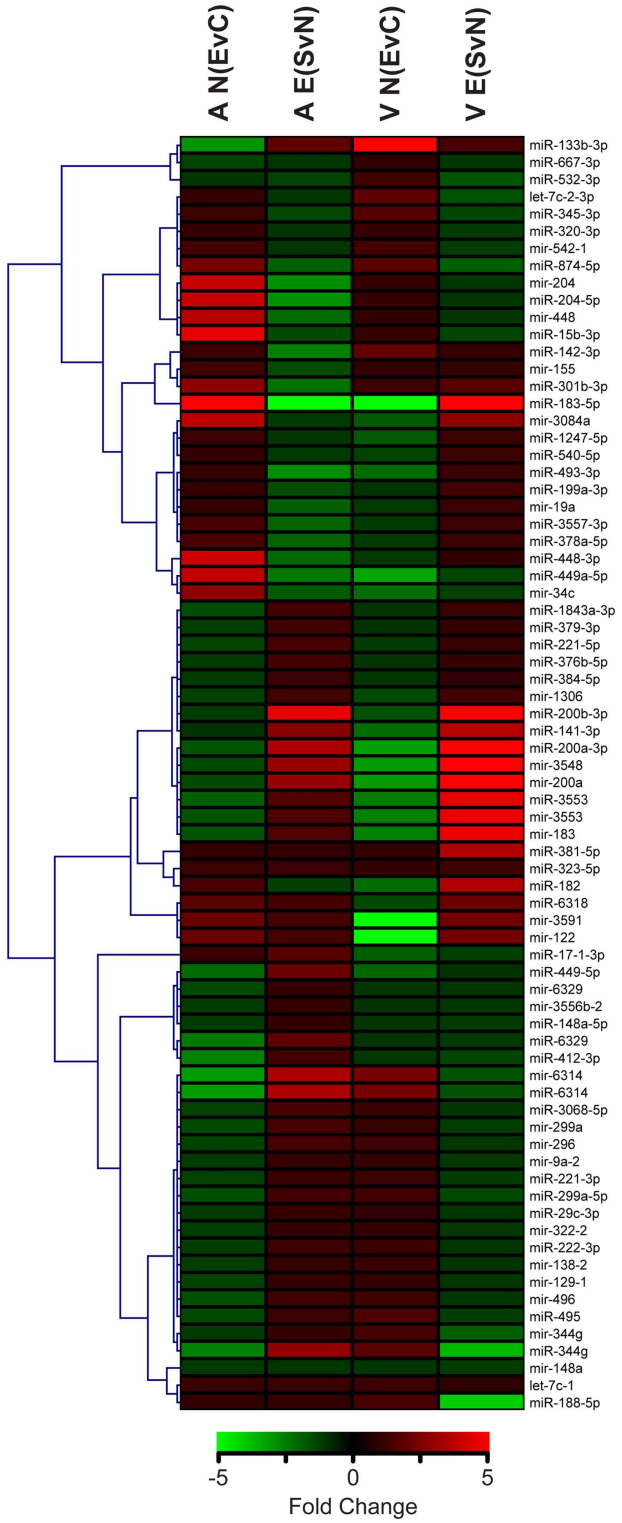

FIGURE 2 | Clustering of microRNAs with ethanol and/or social enrichment effects. Hierarchical clustering analysis of fold change values in miRNAs with significant ethanol effects in non-enriched rats [N(EvC)] and social enrichment effect in ethanol rats [E(SvN)] for both brain regions [A or V].

and thus may be applicable to several miRNAs with the same seed sequence. In this report, we include findings from mRNA targets that have opposing expression level changes to their predicted miRNA regulators (Datasheet S2 in Supplementary Material). Because of their large-scale nature, these results were exclusively examined at the network level.

Ethanol effects reversed by social enrichment

Focusing on the combined miRNA-mRNA target networks in the amygdala, the first major network identified was one involved in cell cycle processes (**Figure 4**, left). This network generally contained RNAs, which were inhibited following prenatal ethanol (e.g., VAMP4, mir-154). Other genes within the network showed increased expression, including p53. Remarkably, almost across the board, the pattern of changes in these genes due to prenatal ethanol was completely opposite the changes seen following social enrichment (**Figure 4**, right).

On the other hand, corresponding ethanol effects in the ventral striatum revolved around cell death processes with inhibition of RNAs including MAP3K2 and upregulation of RNAs like let-7 (**Figure 5**, left). These are predicted to inhibit cell death genes like AKT and ERK1/2. Again, the network appears activated as a result of social enrichment, resulting from downregulation of molecules such as miR-532-3p (**Figure 5**, right).

CANONICAL GENE EXPRESSION PATHWAYS IN NEURONS ARE ALTERED BY ETHANOL AND REVERSED BY SOCIAL ENRICHMENT

In addition to the networks identified above, we also chose to extend our previously reported characterization of 660 brain-related mRNAs of interest (5) by examining the evidence for network level changes in four curated canonical IPA pathways: p53 signaling, GABA receptor signaling, glutamate receptor signaling, and CREB signaling in neurons.

We examined the p53 signaling pathway because of differential responses between prenatal ethanol exposure and social enrichment (**Figure 6**). This network generally showed robust increases in expression following prenatal ethanol exposures in both the amygdala and ventral striatum. Following social enrichment, most of the genes in this network showed decreased expression in the ventral striatum, with a smaller subset showing decreased expression in the amygdala. Thus, the ventral striatum appeared to be more differentially responsive to the social enrichment effects on p53 signaling. Notably, we and others have consistently observed highly consistent changes in p53/apoptosis signaling networks following ethanol exposure (23, 24). However, this is the first report we are aware of to report changes in p53 signaling genes following social enrichment.

We also examined evidence for changes in GABA, glutamate, and CREB signaling. Our rationale for doing so was based on the fact that ethanol acts as a GABA agonist and NMDA antagonist, and has well-characterized effects on CREB signaling within specific brain circuits involved in addiction, including the ventral tegmental area, striatum, and cortex [reviewed in Ref. (25)]. Furthermore, GABA and glutamate neurotransmitter systems have been shown to be differentially expressed in alcohol-preferring vs non-preferring rats (26) and alcohol's effects on the central

Table 3 | Notable miRNA comparisons.

miRNA	miRBase 21 accession	Comparison	RNA-Seq		Microarray	
			Fold change	*p*-Value	Fold change	*p*-Value
DECREASED BY ETHANOL IN BOTH REGIONS						
miR-874-5p	MIMAT0017290	A E(SvN)	−1.91	0.047	−1.28	0.041
miR-874-5p	MIMAT0017290	V E(SvN)	−1.88	0.099	−1.60	0.001
AFFECTED BY SOCIAL ENRICHMENT IN BOTH REGIONS						
miR-183-5p	MIMAT0000860	A E(SvN)	−358.64	0.049		
mir-183	MI0000928	V E(SvN)	4.70	0.026		
MICRORNA-122 FAMILY AFFECTED BY ETHANOL IN VENTRAL STRIATUM						
mir-3591	MI0015471	V N(EvC)	−5.54	0.004		
mir-122	MI0000891	V N(EvC)	−5.54	0.004		
MICRORNA-8 FAMILY AFFECTED BY SOCIAL ENRICHMENT IN VENTRAL STRIATUM						
mir-200a	MI0000943	V E(SvN)	6.81	0.048		
miR-200a-3p	MIMAT0000874	V E(SvN)	7.03	0.041		
miR-200b-3p	MIMAT0000875	V E(SvN)	5.09	0.058	5.45	0.020
miR-141-3p	MIMAT0000846	V E(SvN)	3.60	0.065	1.46	0.010
mir-3548	MI0015404	V E(SvN)	6.81	0.048		
REVERSED BY SOCIAL ENRICHMENT IN AMYGDALA						
mir-204	MI0000946	A N(EvC)	3.97	0.042		
miR-204-5p	MIMAT0000877	A E(SvN)	−2.83	0.084	−3.52	0.057
miR-384-5p	MIMAT0005309	A N(EvC)	−1.12	0.063	−1.21	0.002
miR-384-5p	MIMAT0005309	A E(SvN)	1.12	0.060	1.12	0.038
miR-222-3p	MIMAT0000891	A N(EvC)	−1.23	0.030		
miR-222-3p	MIMAT0000891	A E(SvN)	1.21	0.049		
mir-299a	MI0000970	A N(EvC)	−1.46	0.007		
mir-299a	MI0000970	A E(SvN)	1.41	0.015		
miR-301b-3p	MIMAT0005304	A N(EvC)	2.85	0.017		
miR-301b-3p	MIMAT0005304	A E(SvN)	−2.21	0.041		
miR-6329	MIMAT0025068	A N(EvC)	−2.38	0.003		
miR-6329	MIMAT0025068	A E(SvN)	1.84	0.049		

*Conventions same as **Table 1**. A N(EvC)-ethanol effect in non-enriched rats' amygdala; A E(SvN)-social enrichment effect in ethanol rats' amygdala; V N(EvC)-ethanol effect in non-enriched rats' ventral striatum; V E(SvN)-social enrichment effect in ethanol rats' ventral striatum.*

amygdala are known to affect glutamatergic and GABAergic transmission as a result of acute exposure [reviewed in Ref. (27)].

GABA receptor signaling

In general, we observed trends for decreased expression of multiple GABA related transcripts following prenatal ethanol exposure in both the amygdala and ventral striatum (**Figure 7**, left). These trends were consistently reversed after social enrichment (**Figure 7**, right). These observations suggest a plastic mechanism is in place within the basal forebrain. Markers for GABAergic neurons have been found in the amygdala as early as G20 in rats (28) suggesting that this system could be responsive throughout much of the animal's lifetime.

Glutamate receptor signaling

In contrast to the changes seen for GABA networks, we found evidence of region-specific changes in glutamate related genes. Specifically, prenatal ethanol exposure was associated with generally decreased expression in the amygdala and generally increased expression in the ventral striatum (**Figure 8**, left). However, following social enrichment, both of the brain areas tended to show large-scale increases in expression (**Figure 8**, right). These differences suggest that changes in glutamate signaling in the amygdala may be more directly linked to the social behavioral deficits we have observed, while changes in the ventral striatum may be more reflective of exposure to a drug of abuse. Furthermore, the ethanol findings are also consistent with observations on the acute effects of ethanol on glutamate receptor function [reviewed in Ref. (29)]. Our findings also suggest that glutamate receptor-mediated synaptic plasticity is altered, particularly in the amygdala, consistent with was has been reported for ethanol effects in the hippocampus (30).

CREB signaling in neurons

Reinforcing the changes just described for glutamate signaling, the last network we examined was one involved in CREB signaling in neurons. In this case, we found it much more difficult to generalize about one specific direction of change within this

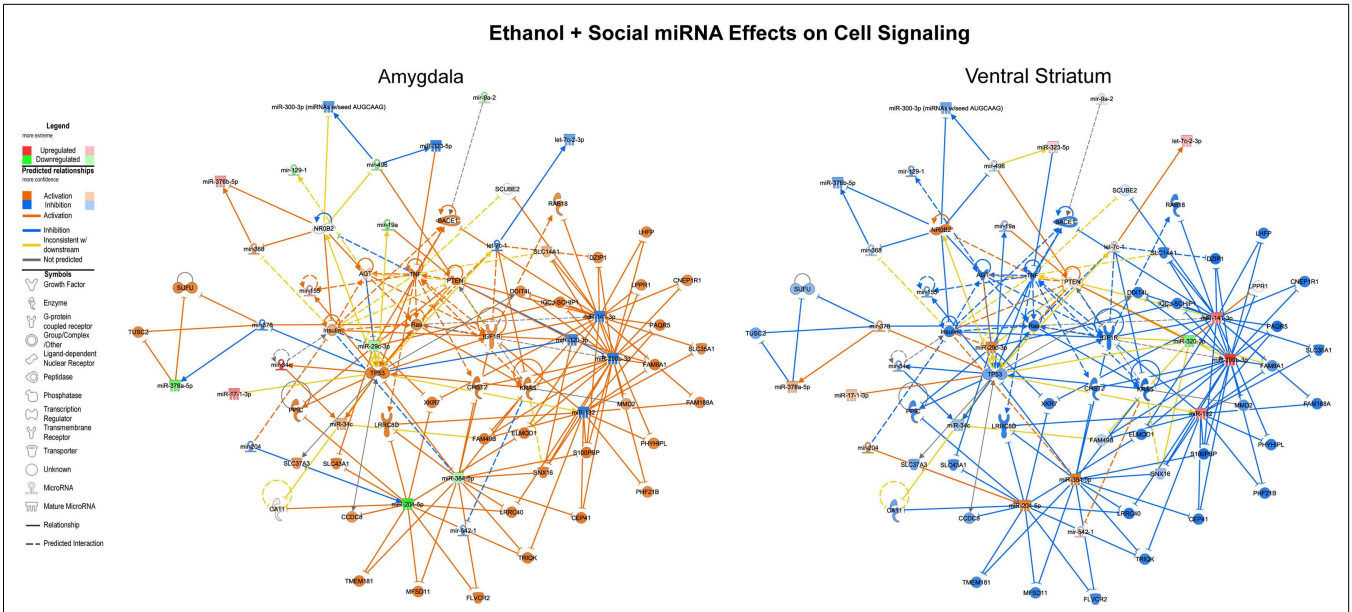

FIGURE 3 | Ethanol and social enrichment effects on microRNAs. The results of a gene network level analysis of all of the miRNAs with changes in one or more of the contrasts listed in **Tables 1** and **2** are shown. Genes and miRNAs with increased or predicted increased expression are shown in red and orange, respectively, while genes and miRNAs with decreased or predicted decreased expression are shown in green and blue, respectively. Genes and miRNAs with conflicting predicted vs observed data are connected by yellow lines, and genes and miRNAs with absent data or unpredicted relationships are unfilled and connected by gray lines. Note that in general, the network is activated in the amygdala and inhibited in the ventral striatum.

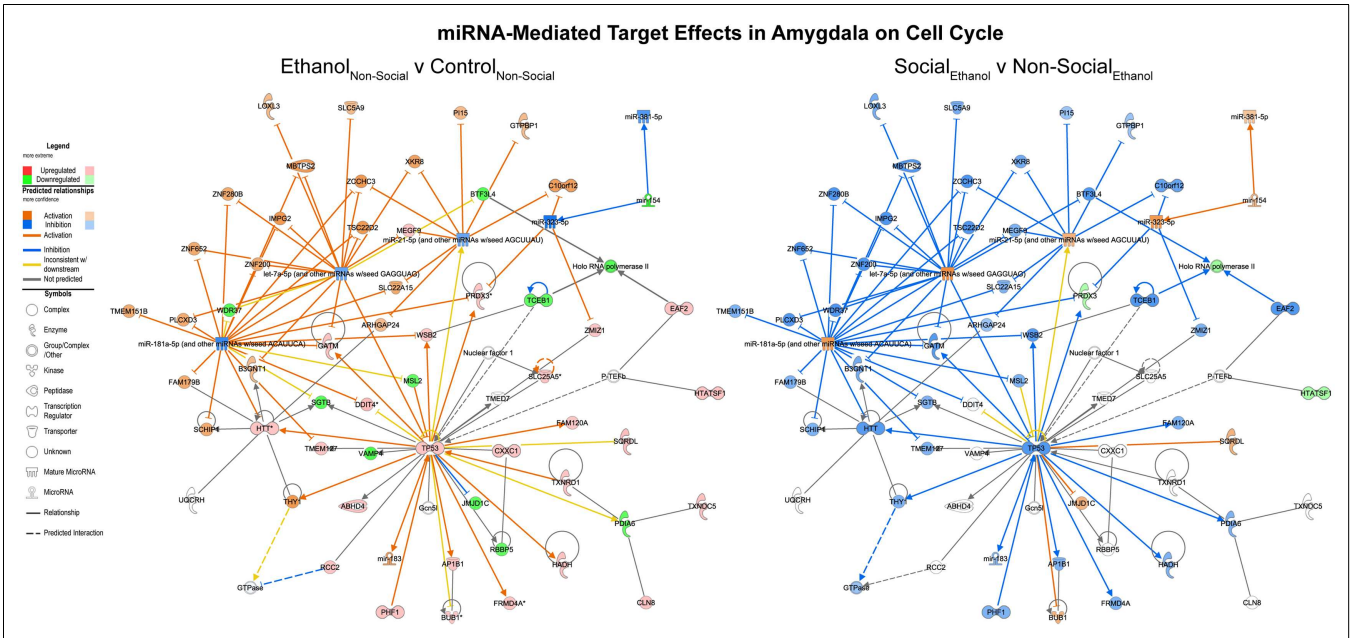

FIGURE 4 | MicroRNA target effects in the amygdala on cell cycle signaling. Conventions same as **Figure 3**. Note that nearly all of the genes show a distinct reversal in their expression difference following social enrichment.

highly integrated cellular network across the two brain areas. Indeed, following prenatal ethanol exposure, more than 10 genes showed changes in distinctly opposite directions in the amygdala and ventral striatum (e.g., IGLUR, Gβ, Gγ, PLC, PKC, AKT, ERK, p90RSK, p300, CBP, TFIIB, TBP) (**Figure 9**, left). Following social enrichment, however, there was somewhat greater agreement between the two brain areas in the directionality (or predicted directionality) of the changes (**Figure 9**, right). These

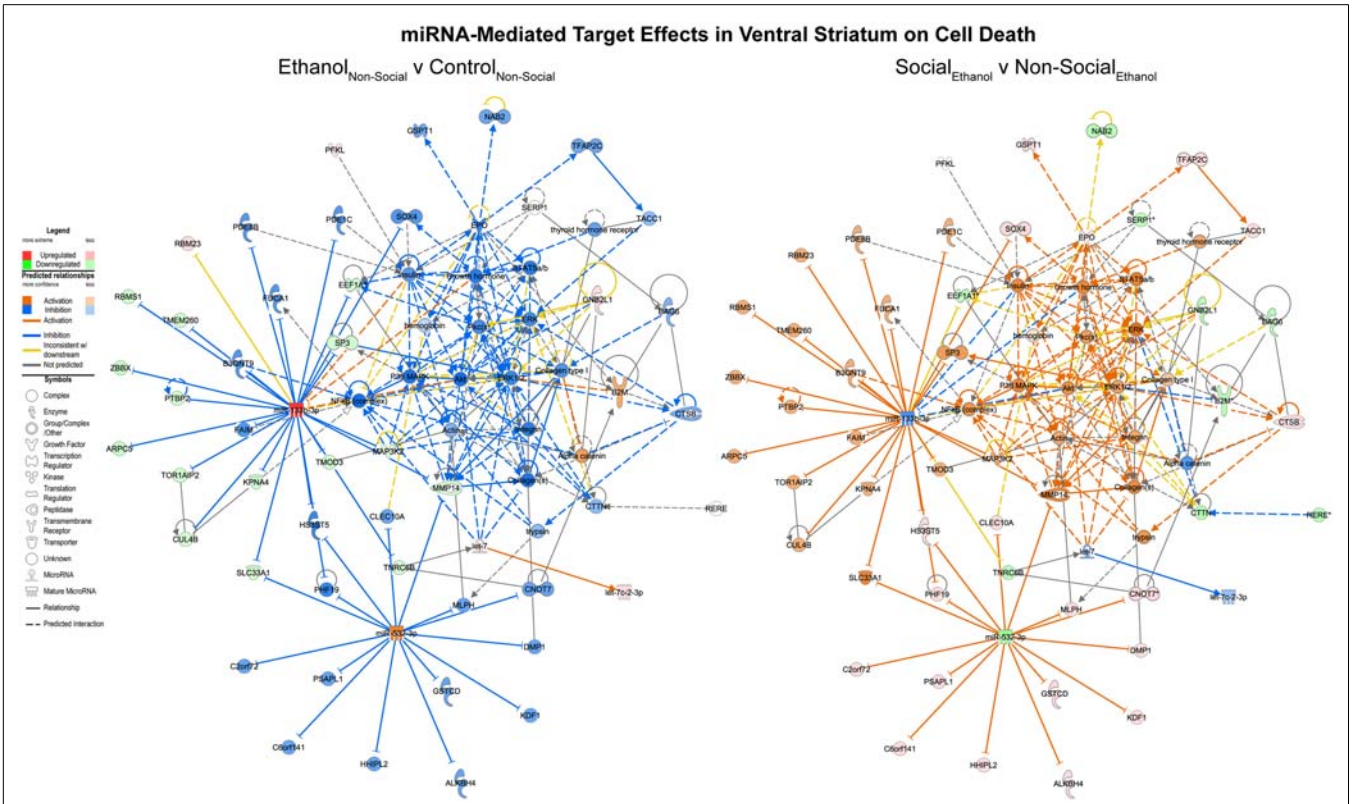

FIGURE 5 | MicroRNA target effects in the ventral striatum on cell death signaling. Conventions same as **Figure 3**. Note that nearly all of the genes show a distinct reversal in their expression difference following social enrichment.

findings underscore the importance of examining entire transcriptional networks before reaching conclusions regarding the potential effect that a manipulation or treatment may have in a specific brain region. Moreover, the results highlight the utility of examining multiple brain regions.

DISCUSSION

This study sought to evaluate molecular mechanisms at the miRNA, mRNA, and gene regulatory network levels that underlie the effective reversal of a social motivation deficit seen following prenatal ethanol exposure (5). We found several robustly affected miRNAs, target mRNAs, and functional pathways that could represent candidate control points for the behavioral deficits we previously observed.

Several recent miRNA studies have been performed on rodents or primary cell cultures exposed to ethanol either during gestational or postnatal time periods. We compared the most robustly changed miRNAs in our studies (**Tables 1** and **2**) to results from these other studies. Interestingly, 24 of the 48 miRNAs we observed with changes due to ethanol in either the amygdala or ventral striatum were also reported to change in other studies. Of those, 9 miRNAs (let-7c-1, miR-221-3p, miR-221-5p, miR-222-3p, mir-322-2, mir-34c, miR-384-5p, mir-496, and mir-542-1) reported consistent directional changes as our data (15, 31–33). This is despite the use of different exposure paradigms, brain areas, and cell types, as well as different species. These miRNAs may thus represent highly robust and persistent indices of ethanol exposure.

Most importantly, two of these microRNAs (miR-222-3p and miR-384-5p) were also found to be reversed in the amygdala after social enrichment (**Table 3**). By targeting the PTEN gene, mir-222 has been shown to promote neurite outgrowth (34). Additionally, mir-384 has been shown to be an indicator of neurotoxicity (35) and was found to be differentially expressed in dopaminergic neurons following cocaine addiction (36).

On the other hand, 7 miRNAs (mir-138-2, miR-148a-5p, mir-299a, miR-299a-5p, miR-493-3p, miR-540-5p, and miR-667-3p) have reported significant changes in miRNAs that only show directional changes opposite to what we observed (31, 32, 37, 38). Finally, there is mixed support for 7 of the miRNAs we reported (mir-129-1, miR-15b-3p, mir-204, miR-29c-3p, miR-301b-3p, miR-495, and mir-9a-2), with some studies showing changes consistent with our data, and other studies showing changes opposite those of our study (15, 31–33, 38–47). It is important to note that mir-9 has well-established roles in neurogenesis [reviewed in Ref. (48)]. Possible explanations for the disparities in results are likely found in the parameters of those studies. Regardless, these latter two sets of miRNAs may represent less-reliable or less persistent biomarkers of ethanol exposure.

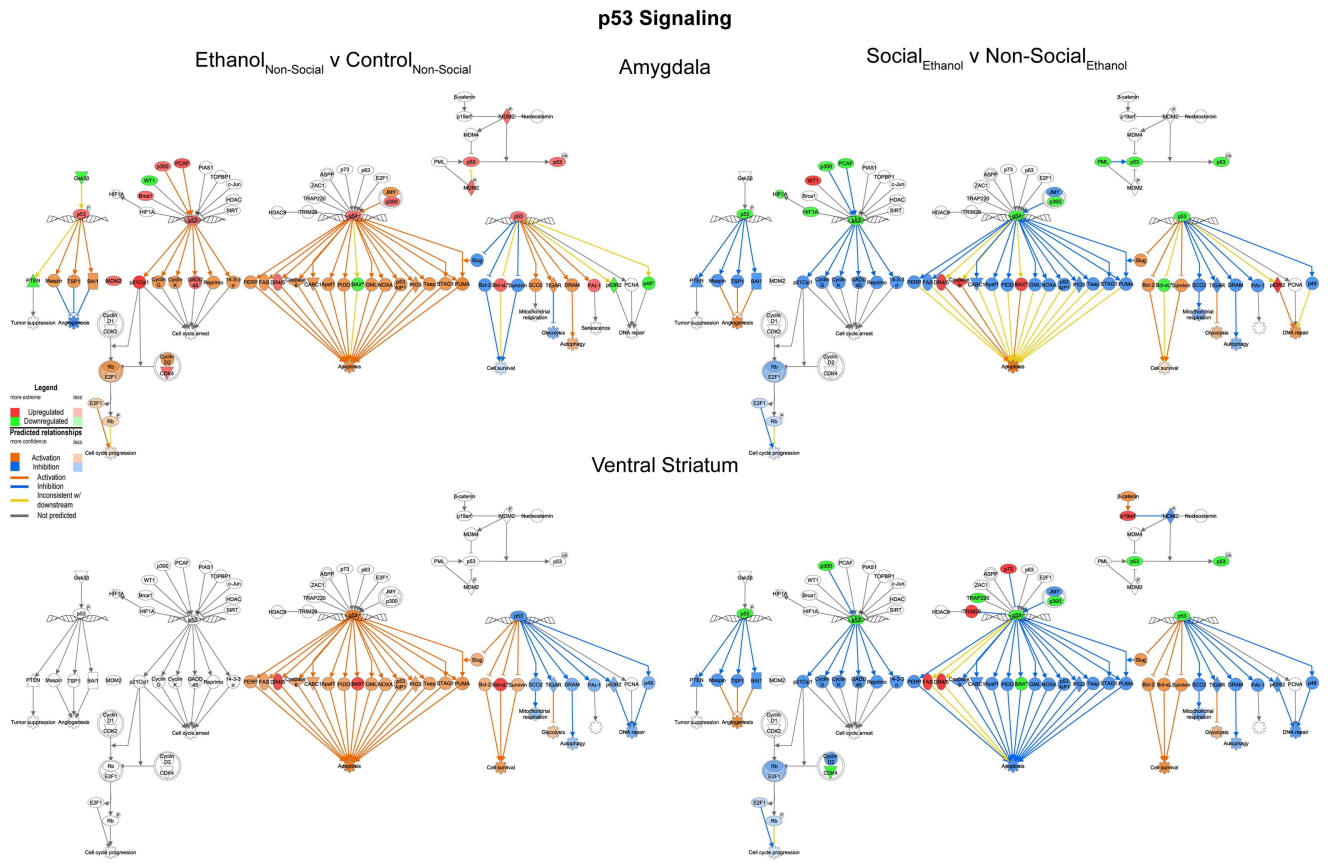

FIGURE 6 | Ethanol and social enrichment effects on nuclear p53 signaling. Conventions same as **Figure 3**. Note that the changes in p53 signaling were highly similar in both brain areas following prenatal ethanol exposure (left), but showed differential responsiveness in the two brain areas following social enrichment (right).

Rather than focusing on individual genes, our large-scale analyses of ethanol-induced changes in miRNA and mRNA expression focus on whether effects on functionally related pathways are consistently seen across studies. From this perspective, our results add substantial support to the concept of a systems-level disruption of major gene regulatory pathways by a common environmental insult. We show in our study that prenatal alcohol exposure imposes a long-lasting effect on neuronal and, ultimately, behavioral function in adolescents. Notably, our results also extend our previous molecular analyses by incorporating the vast post-transcriptional regulatory aspects embodied by microRNAs. By evaluating concurrent changes in miRNA and mRNA levels, this work shines light on an additional layer of complexity to the gene expression changes occurring in the amygdala and ventral striatum. This is critical because miRNAs are thought to respond greatly to environmental stressors and are thought to mediate global gene expression changes [reviewed in Ref. (49, 50)].

Notably, the functions represented by the pathways represented in **Figures 3–5** clearly implicate alterations in p53 signaling, cell cycle, and cell death pathways as a consequence of prenatal ethanol exposure. These pathways are of particular note because they suggest that abnormal cellular proliferation and/or DNA damage repair processes could be associated with early ethanol exposures. Indeed, we previously reported robust changes in genes involved in these processes in adult human alcohol abusing subjects (23). Although there is evidence that cortical heterotopias can occur as a consequence of early ethanol exposure, data from several human studies have failed to demonstrate any consistent elevation in the risk for childhood cancers, with some studies even reporting protective effects (51, 52).

To our knowledge, this is the first report of miRNA-directed gene expression changes brought about by environmental interventions in any FASD model. The potential reversal of abnormal changes in miRNA and mRNA expression by a relatively simple intervention (social enrichment) is consistent with data from other disorders, where specific changes in miRNA levels have been seen to result from an enriched environment, corresponding with slowing of the disease progression and improvement in hippocampal synaptic transmission using an Alzheimer's disease model (53). Given the considerable data showing that environmental enrichment is likely one of the most effective means of improving outcomes in children with FASD and autism spectrum disorders, it is highly likely that such interventions exert at least some of their therapeutic effects through alterations in miRNA and mRNA levels in some of the same brain circuits we examined in this report.

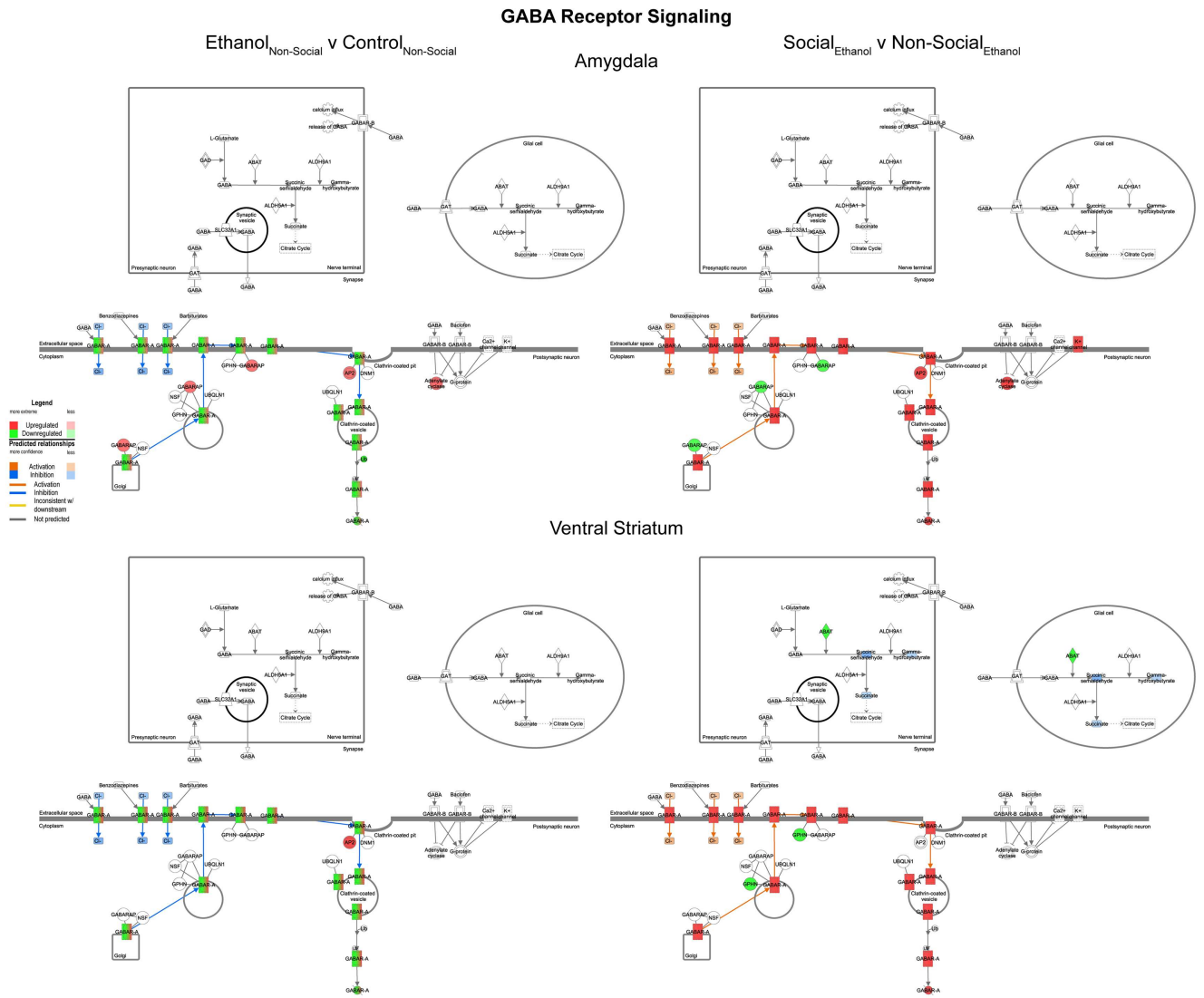

FIGURE 7 | Ethanol and social enrichment effects on GABA receptor signaling. Conventions same as **Figure 3**. Note that prenatal ethanol exposure generally inhibits GABA signaling and is reversed following social enrichment. In some cases, due to the presence of multiple isoforms of a gene with the same name, multiple colors are contained within a symbol.

It is beyond the scope of the present report to fully examine the evidence for regionally specific changes in expression. However, we note that both at the individual miRNA level and target mRNA level, many of the changes appeared to be region specific. This was even more evident for some of the comparisons made for specific functional pathways. Taken together, these patterns reinforce other recent findings, such as those by Tapocik et al. (54), who showed that mir-206 upregulation due to ethanol is regionally selective in the medial prefrontal cortex of a rat model of alcohol dependence and is not found in the amygdala or other regions of the brain. Clearly, much additional work will be needed to create comprehensive profiles for all of the brain-wide changes seen following prenatal ethanol exposure or social enrichment.

There are several limitations to note in the present study. First, it is important to acknowledge that the resulting gene expression effects we have observed in P42 rat brains reflect the cumulative effect of all life experiences to that age. That is, everything that the animal has experienced could alter miRNA and mRNA expression patterns; consequently, some of these alterations could interact with variables that were outside our ability to control. We tried to minimize the differences due to random noise that might exist between treatment groups. Nonetheless, in our study, pregnant animals ware received in the lab on G4, handled and injected on G12, potentially causing gestational stress. Exposure of the offspring to the anesthetic agents ketamine and xylazine immediately prior to decapitation may also have altered gene expression in some manner. It is possible that the treatment itself (ethanol exposure)

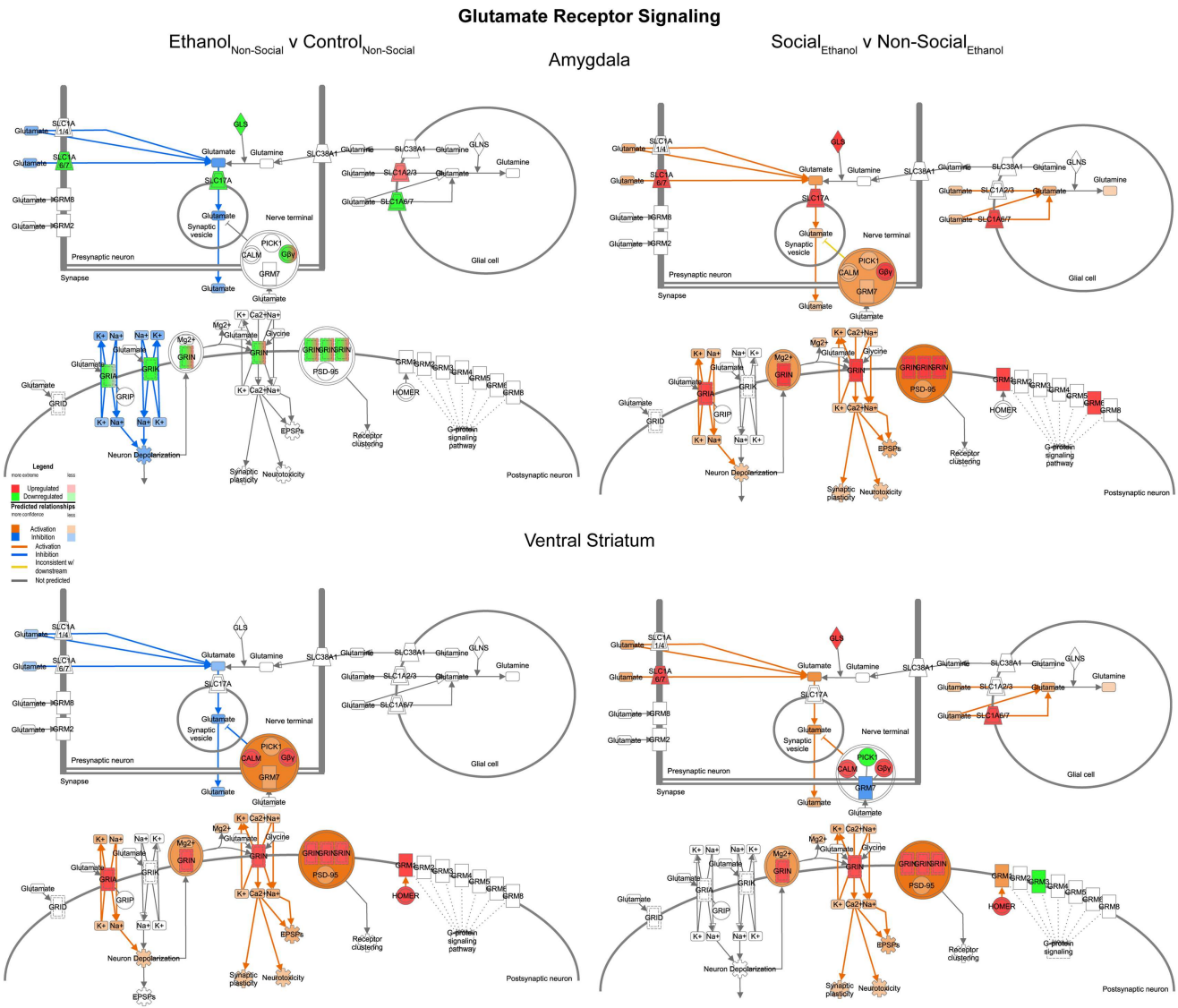

FIGURE 8 | Ethanol and social enrichment effects on glutamate receptor signaling. Conventions same as **Figure 3**. Note that this network tended to show differential changes due to prenatal ethanol exposure in the two brain areas (left), but somewhat more consistent changes following social enrichment (right).

interacted with the pre- or postnatal stressors, which include shipment, handling, and anesthetic administration. We note that the time course by which the anesthetics acted (ketamine/xylazine) is extremely brief (lasting only a few minutes prior to decapitation and dissection) and thus is not likely to create any large-scale biases in gene expression differences.

Another limitation in the present study is the lack of correction for multiple testing. We contend that our use of two independent quantification methodologies somewhat mitigates this concern. Furthermore, our focus on functional gene network analysis, rather than individual miRNAs and mRNAs *per se* also helps reduce concern about type 1 error. The seemingly low concurrency of RNA-Seq and array data may be the result of several factors, most notably the use of 2 different miRBase databases in our high-throughput quantification (array used miRBase 15, while sequencing uses miRBase 21). In addition, we used very stringent concurrency criteria because it was based on exact sequence homology between the array probe and the gene annotation against which the RNA-Seq data were quantified upon.

In conclusion, despite some limitations, our data strongly demonstrate that prenatal ethanol exposure has the capacity to impart long-lasting gene expression changes at both the miRNA and subsequent target mRNA level. Some of these changes clearly impact large-scale functional pathways in the brain that are involved in synaptic function and intracellular signaling, as well as cell cycle regulation, and brain development. Further studies are necessary to determine the extent to which changes in these pathways represent points of no return, or novel therapeutic opportunities for intervention.

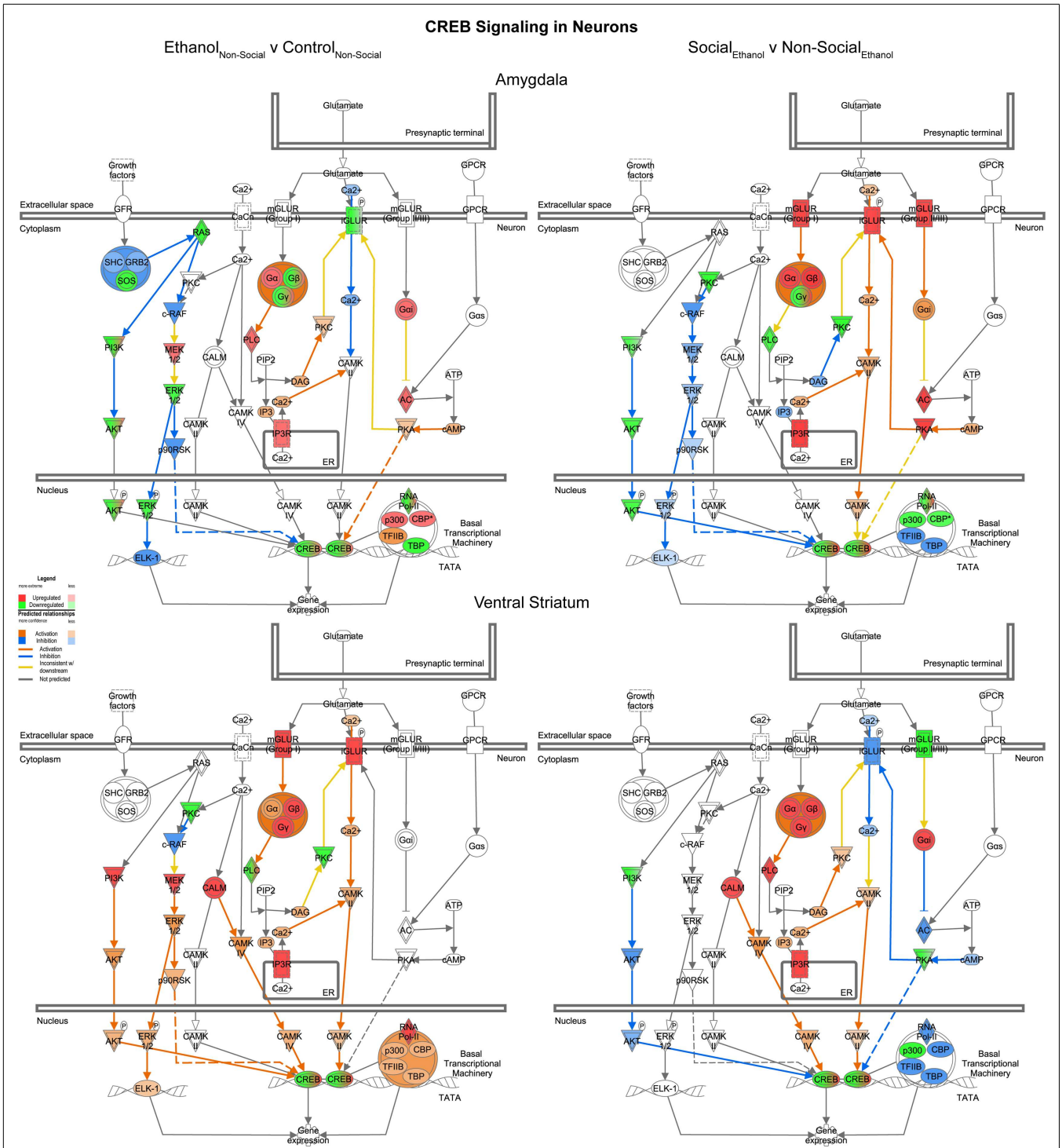

FIGURE 9 | Ethanol and social enrichment effects on CREB signaling in neurons. Conventions same as **Figure 3**. Note that the changes produced following prenatal ethanol exposure or social enrichment showed somewhat complex patterns, with some evidence of both region-specific and treatment-specific effects.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fped.2014.00103/abstract

Datasheet S1 | *p*-values for main and interaction effects in miRNAs of interest. These include miRNAs with significant ethanol effects in non-enriched rats [N(EvC)] and social enrichment effect in ethanol rats [E(SvN)] from both brain regions.

Datasheet S2 | Messenger RNA targets from group comparisons. Targets were filtered on opposing expression pairing between miRNAs and corresponding mRNA targets. Genes with functions related to neurological disease are highlighted in green. Microarray and RNA-Seq expression data have been deposited in the NCBI Gene Expression Omnibus (GEO accession # GSE60901, which includes microarray subseries GSE60819 and RNA-Seq subseries GSE60900).

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Visual defects in a mouse model of fetal alcohol spectrum disorder

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Alcohol consumption during pregnancy can lead to a multitude of neurological problems in offspring, varying from subtle behavioral changes to severe mental retardation. These alterations are collectively referred to as Fetal Alcohol Spectrum Disorders (FASD). Early alcohol exposure can strongly affect the visual system and children with FASD can exhibit an amblyopia-like pattern of visual acuity deficits even in the absence of optical and oculomotor disruption. Here, we test whether early alcohol exposure can lead to a disruption in visual acuity, using a model of FASD to mimic alcohol consumption in the last months of human gestation. To accomplish this, mice were exposed to ethanol (5 g/kg i.p.) or saline on postnatal days (P) 5, 7, and 9. Two to three weeks later we recorded visually evoked potentials to assess spatial frequency detection and contrast sensitivity, conducted electroretinography (ERG) to further assess visual function and imaged retinotopy using optical imaging of intrinsic signals. We observed that animals exposed to ethanol displayed spatial frequency acuity curves similar to controls. However, ethanol-treated animals showed a significant deficit in contrast sensitivity. Moreover, ERGs revealed a market decrease in both a- and b-waves amplitudes, and optical imaging suggest that both elevation and azimuth maps in ethanol-treated animals have a 10–20° greater map tilt compared to saline-treated controls. Overall, our findings suggest that binge alcohol drinking restricted to the last months of gestation in humans can lead to marked deficits in visual function.

Keywords: retinotopy, fetal alcohol spectrum disorders, fetal alcohol syndrome, visual evoked potentials, optical imaging of intrinsic signals, electroretinography, vision, visual cortex

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term for a variety of conditions affecting the children of women who drink alcohol during pregnancy and is currently one of the leading causes of mental retardation in the world. The effects of early alcohol exposure are wide ranging and can vary from subtle behavioral changes to severe cognitive deficits. Sensory processing deficits may exacerbate the neurobehavioral problems observed in FASD, as these subjects often exhibit delays in auditory processing as well as reduced vision acuity (1, 2).

A subgroup of FASD is Fetal Alcohol Syndrome (FAS), which is characterized by the triad of growth deficiency, central nervous system (CNS) problems, and a specific pattern of facial dysmorphology (3–5). The altered facial features, which include small (or lack of) philtrum, short nose, flat midface, and low nasal bridge are caused by a specific effect of alcohol during the gastrulation phase of development (6–8).

The visual system can be particularly affected by developmental alcohol exposure. Children with FASD often present deficits in spatial frequency acuity and contrast sensitivity, strabismus, amblyopia, poor detection of geometric designs, and abnormal saccadic movements (1, 9–13). The type of visual deficit observed is strongly related to the time of alcohol exposure. Similar to the typical FAS facial dysmorphology, gross ocular defects such as microphthalmia and hypoplasia of the optic nerve are caused by alcohol exposure during the first trimester of the human gestation (6, 8, 14).

Hug and colleagues evaluated the visual abilities of a group of children with FAS (15). All the subjects displayed facial dysmorphology and 10 out of 11 had hypoplasia of the optic nerve. They found that kids with FAS showed a reduction in spatial frequency acuity measured by visually evoked potentials (VEPs) and reduced a- and b-waves after electroretinography (ERG) (15). Studies using animal models have been instrumental in investigating the effects of alcohol consumption at different times during pregnancy, corresponding to different periods of fetal brain development (7). For instance, Katz and Fox demonstrated, in an animal model, findings similar to the human study mentioned above (16). They evaluated

ERGs in two paradigms of developmental alcohol exposure. In one experimental group, alcohol exposure (liquid diet, 35% alcohol) lasted from the first day of the pregnancy to parturition (roughly equivalent to the first, and most of the second trimester of human gestation). In another experimental group, alcohol exposure began at the first day of pregnancy but was extended to postnatal day 10 (mimicking exposure all through human gestation). Both groups showed a decrease in the amplitude of a- and b-waves in ERGs. These deficits were more evident in low-light conditions (scotopic vision) (16).

While major ocular malformations have clear effects in visual perception, it is not uncommon for children with FASD to show reduced visual function even when these malformations are not evident. In fact, Vernescu and colleagues were able to detect deficits in visual acuity and contrast sensitivity in children with FASD even in the absence of refractive errors (1).

The last months of gestation in humans and the first two postnatal weeks in rodents are crucial for visual system development. During this period, the retina displays "waves" of activity, which are important for the establishment of the topography of the retinogeniculo-cortical pathway (17–20). Also during this period, the retina and most of the CNS are extremely sensitive to alcohol-triggered neuroapoptosis (21, 22). Therefore, we hypothesize that alcohol exposure even restricted to just this period of development will affect visual function. Our prediction is that alcohol exposure in mice during the first two postnatal weeks will disrupt their spatial frequency acuity and contrast sensitivity measured by VEPs; reduce amplitude of a- and b-waves of ERGs and alter cortical retinotopic maps assessed by optical imaging of intrinsic signals.

MATERIALS AND METHODS

All procedures described in this paper were approved by the Institutional Animal Care and Use Committee.

ALCOHOL EXPOSURE PARADIGM

Visibly pregnant C57/BL6 mice were obtained from a commercial supplier (Harlan), and singularly housed in the university animal housing. Pregnant dams were checked daily until pups were born. Day of birth was designated as postnatal day (P) 0.

We used the same paradigm of alcohol exposure used in a recent study by our lab showing that developmental alcohol exposure leads to impaired visual cortex plasticity in mice (23). Pups received a single injection of 5 g/kg of alcohol (25% ethanol in normal saline i.p.) or an equivalent volume of saline as a control on days P5, 7, and 9. Typically, within a litter (males and females) 2/3 of animals were injected with alcohol and the remaining with saline. Animals were then alcohol-free for the remainder of the study. According to our previous studies, this exposure paradigm leads to blood alcohol levels of 411 mg/dl (1) at 1 h post injection.

VISUALLY EVOKED POTENTIALS

Surgery

Visually evoked potentials are assessed in awake mice through the use of chronic implanted electrodes. For electrode implantation, P21–22 mice were anesthetized with ketamine (120 mg/kg (Bioniche Pharma, Lake Forest, IL, USA) and xylazine 9 mg/kg (Akorn, Inc., Decatur, IL, USA). Once anesthetized, 2% lidocaine jelly (Akorn, Inc., Decatur, IL, USA) was applied locally on the scalp at the incision site. Silver ground electrodes were implanted 1.0 mm caudal from bregma, and 2.0 mm lateral from the midline.

Tungsten microelectrodes (FHC, impedance 0.3–0.5 MΩ) were implanted 3.00 mm lateral of the midline and 0.5 mm of lambda, at a depth of 0.43 mm. Electrodes were secured to the skull with cyanoacrylate glue (Elmers, Westerville, OH, USA). After surgery, the animal was monitored until recovery of righting reflexes and was then given 0.05 mg/kg of buprenorphine (Stokes Pharmacy, Mt. Laurel, NJ, USA) for post-surgical analgesia.

Recording

After the implantation of the electrodes, animals were allowed to recover for 48–72 h. After the recovery period, awake animals were habituated on the experimental setup for 45 min 1 day prior to the experiment. VEPs were recorded using XCell-3 amplifiers (FHC, Inc., Bowdoin ME, USA; one for each recording electrode), a 1401 digitizer (CED, Cambridge, England), and Spike 2 software (Cambridge Electronics Design, Cambridge, England). Visual stimulations were presented to each eye individually using a monitor placed 18 cm from the nose of the animal (mean luminance 27 cd/m^2, area of 15 cm × 31 cm) and controlled by a custom program using MATLAB (MathWorks, Natick, MA, USA) with Psychtoolbox extensions.

VISUAL STIMULI AND ANALYSIS

Stimuli consisted of full-field ordinal sine-wave 2 Hz reversing gratings, at 0.05 cycles per degree (cpd) with 100% contrast. VEP measurements were based on the average amplitude of 100 stimulation presentations. Recorded VEP amplitudes were then used to calculate a contralateral bias index (CBI, ratio of contralateral/ipsilateral response amplitude of each animal). CBI results are reported as average CBI and the standard error of the mean (SEM). For spatial frequency acuity measurements, the stimuli consisted of six randomized full-field reversing sine-wave gratings of 0.5–0.02 cpd and an equal luminance gray screen. Spatial frequency acuity measures were based on average VEP amplitude of 100 trials. For contrast sensitivity measurements, six randomized full-field reversing sine-wave gratings, of 0.05 cpd, with equal luminance and contrasts from 100 to 0% were presented. Contrast sensitivity acuity measures were based on average VEP amplitude of 100 trials. All acuity results are reported as the average VEP amplitude in millivolts and the SEM.

ELECTRORETINOGRAPHY

Mice were dark adapted overnight and prepared for recording the next day under infrared illumination. Animals were anesthetized with a mixture of ketamine/xylazine (150/10 mg/kg; IP), and the pupils were dilated in the dark for a minimum of 10 min with topical eye drops of 1% tropicamide and 2.5% phenylephrine (Bausch & Lomb, Tampa, FL, USA). The head was held steady in a custom nose ring. A drop of 0.9% saline was frequently applied on the cornea to prevent dehydration, also allowing electrical contact with the recording electrode (a gold wire loop). A sterile reference needle electrode (Rhythmlink, Columbia, SC, USA) was inserted under the caudal most portion of scalp, behind the VEP headstage. Amplification (at 1–500 Hz bandpass, without notch filtering), stimuli presentation, and data acquisition were programed

and performed using the UTAS-E 3000 system (LKC Technologies, Gaithersburg, MD, USA) as previously described (24). Scotopic ERG responses were recorded during single 10 microseconds flash presentations at intensity of 1.37 log cds/m^2. Six responses were obtained at 20-s intervals, and were averaged for each eye. All results are reported as the average response amplitudes in millivolts and SEM.

OPTICAL IMAGING OF INTRINSIC SIGNALS

Surgery

Mice between 25 and 50 days of age were anesthetized with 10% urethane in saline (1.0 g/kg) injected intraperitoneally. A supplementary sedative, chlorprothixene (0.2 mg/mouse i.p.) was administered prior to urethane. Atropine (5 mg/kg) was injected subcutaneously to reduce bronchial secretion and to counteract the parasympathomimetic effect of the anesthetic agents. An incision was made in the scalp, exposing the occipital region of the animal's skull. A metal plate with a square window in the center was then glued to the skull, positioning the square window above the visual cortex. Agarose (2.5% in saline) was used to fill the square window and topped with a glass coverslip. A craniotomy is not required with this technique because the mouse's skull is sufficiently transparent for clear imaging.

Image processing

The acquired images were used to create the retinotopic maps; the procedures were similar to those described in Kalatsky and Stryker (25). In short, the time series of light reflectance from each pixel was analyzed independently, after filtering (high-pass, boxcar filter, and size two cycles of the stimulus) the fundamental Fourier component was extracted at the frequency of stimulation. These phases and amplitudes were computed from the cosine and sine components and were used to create the maps. The phase maps are the maps of relative retinotopy. To remove the constant bias, a small region away from the visual evoked activity and free of the vascular and other artifacts was selected, and the mean value of cosine and sine components was computed, this two-dimensional vector was subtracted from all pixels of the map. The maps produced by the oppositely moving stimuli (up–down and right–left) were combined, the phase of each pair of corresponding pixels were subtracted and the amplitude average, to yield the maps of absolute retinotopy (elevation and azimuth) see **Figure 4A**. Finally, to yield maps of the visual angle the phase maps were divided by a constant factor (7.2).

Analysis of retinotopic maps

Although the measure used in this study (map tilt, phase scatter, and magnification factor) do not require the absolute value maps we favored this approach because the combined maps have higher signal-to-noise ratio and for easy of comparison to other studies (26).

Map tilt. The map tilt was computed as the angle of the representation of the central line of the stimulus monitor on the cortex. The central line (horizontal for the up–down stimulus and vertical for the right–left stimulus) has zero-phase in the absolute retinotopic maps, which is coded in color blue (**Figure 4**). The angle of the zero-phase line on the cortical surface was computed relative to the mediolateral axis for the elevation maps and the anteroposterior axis for the azimuth maps. Results are reported as the mean angle and SEM.

Phase scatter. To evaluate the quality of the maps we used the phase scatter, which was computed as the difference between the phase of a pixel and the mean phase of its neighborhood (including the pixel itself). The neighborhood was defined as the pixels within a circle of radius 2.9 drawn around the central pixels, which resulted in 5×5 square footprints. A region with visually evoked activity and free of artifacts was selected. The pixels within the region were ranked by amplitude of response and at most 20,000 pixels within the highest response were selected. The standard deviation of the phase scatter of these pixels was used as the measure of the map quality.

Magnification factor. The magnification factor (the amount of visual angle representation per millimeter of cortical distance) was computed along the lines perpendicular to those used for the map tilt calculation (the zero-phase lines), these lines correspond to the steepest progression of the phase change. Two points were selected on the steepest phase ascent line corresponding to approximately $\pm 5°$ of the visual angle; the cortical distance between these two points was measure (in millimeters). The ratio of the visual angle difference (typically 10 degrees) over this distance yields the magnification factor. Results are reported as the average visual degree per millimeter and the SEM.

RESULTS

VEP AMPLITUDES

At P25, contralateral bias was assessed by peak to trough measures of VEPs resulting from stimulation of each eye individually. Saline-treated animals exhibited the expected contralateral eye dominance with average contralateral bias indexes (CBIs, ratio of contralateral/ipsilateral response amplitude of each animal) of 1.6 ± 0.1 ($n = 12$). In this group, the average amplitude of contralateral and ipsilateral eye responses were 197.2 and 127.8 mV $\pm$ 18.5, respectively. Animals exposed to early alcohol exposure demonstrated an average CBI value of 1.5 ± 0.1 (**Figure 1A**) similar to saline controls. Yet, early ethanol exposure affected the strength of VEPs as alcohol-treated animals showed significantly lower amplitudes than controls in response to either contralateral (120.2 mV $\pm$ 8.6; $t = 3.6$, $p < 0.01$, df = 19) or ipsilateral (83.3 mV $\pm$ 7.1; $t = 2.25$; $p < 0.05$; df = 18) eye stimulation (**Figure 1B**).

VISUAL ACUITY

Figure 2A shows responses to gratings from 0.02 to 0.50 cpd. Responses were normalized to the amplitude of the response to 0.02 cpd. Saline and ethanol-treated animals demonstrated similar spatial frequency acuity curves with maximal responses at 0.02 and 0.05 cpd, which decreased until responses could not be detected above noise at 0.50 cpd. In fact, a repeated measures ANOVA showed no differences between-groups for saline and ethanol-exposed animals ($F = 0.6$, df = 1, $p = 0.4$), but there was a significant linear effect within subjects ($F = 331.2$, df = 1,

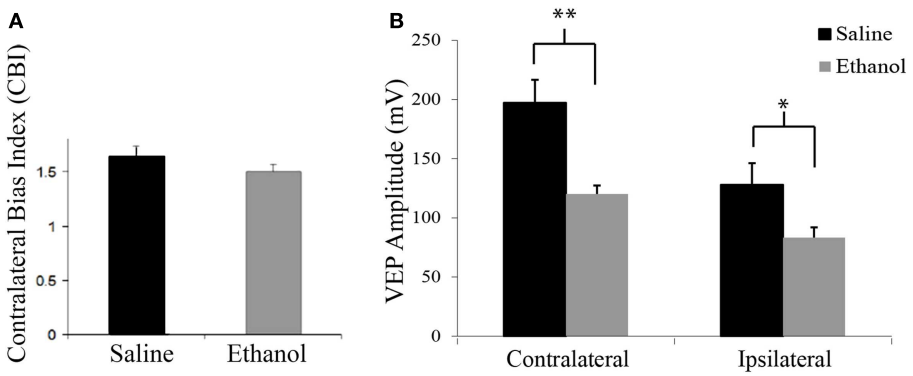

FIGURE 1 | Contralateral bias index and amplitudes of visually evoked potentials. **(A)** Alcohol treatment does not affect the eye dominance in the binocular zone of the visual cortex. Note similarity of CBIs between groups. **(B)** Alcohol treatment affects amplitude of visually evoked potentials. Note that VEPs elicited by either contralateral or ipsilateral eye stimulation resulted in lower amplitude values in the ethanol group (contra: 127.82 mV ± 18.46; ipsi: 83.31 mV ± 7.09) than the saline group (contra: 197.16 mV ± 19.5; ipsi: 120.17 mV ± 8.61). $*p < 0.05$; $**p < 0.01$.

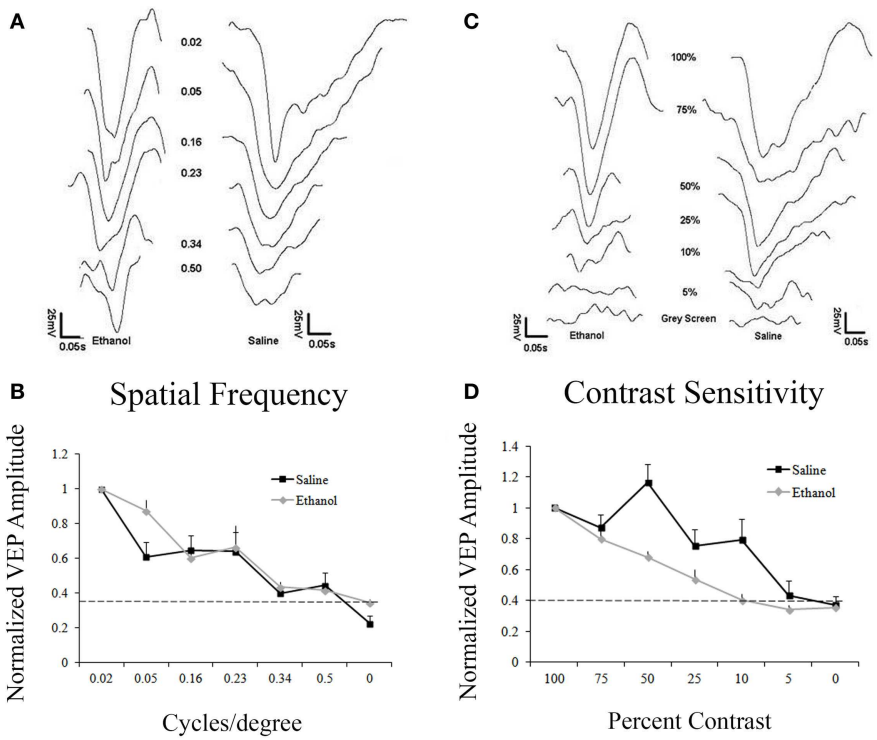

FIGURE 2 | Responses to varying contrast and spatial frequency. **(A)** Representative VEP traces from an ethanol and a saline-treated animal after stimulation with different spatial frequencies (cycles/degree, cpd). **(A,B)** Saline and ethanol-treated animals showed similar decay in amplitudes after decreasing spatial frequencies were presented. Minimal responses at 0.5 cpd. Dotted line represents noise. **(C)** Representative VEPs from an ethanol and a saline-treated animal after visual stimulation at different contrasts. **(C,D)** As contrast decreases, saline-treated animals have a slow decrease in responses, showing a small response to 5% contrast, while ethanol-treated animals sharply drop-off their responses after 100% contrast, with no response occurring at 10% contrast.

$p < 0.001$), indicating differences in response amplitude compared to changes in the cpd for each stimulation (**Figure 2B**). These results are compatible with the spatial frequency acuity responses described for mice (27).

Despite no change in spatial frequency acuity, we decided to explore contrast sensitivity in saline and ethanol-exposed animals. Animals were presented with 0.05 cpd stimuli with different levels of contrast from 100 to 5%. Contrast sensitivity responses

were normalized to the amplitude of the response to 100% contrast. Control animals exhibited contrast sensitivity with peak responses occurring at 100 and 75% contrast. Response amplitudes then slowly decreased until there was barely any response above noise at 5% contrast. In contrast, ethanol-treated animals exhibited a precipitous drop-off of responses after 75% contrast, with no response detectable above noise at 10% contrast (**Figure 2C**). When this difference in contrast sensitivity was compared using a repeated measures ANOVA, there was a significant linear effect within subjects ($F = 428.7$, df $= 1$, $p < 0.001$), indicating differences in response amplitude compared to changes in contrast. Moreover, in between-groups measures, ethanol-exposed animals were shown to be significantly different from their control counterparts ($F = 7.7$, df $= 1$, $p < 0.05$) (**Figure 2D**).

ELECTRORETINOGRAPHY

ERG was assessed in 12 saline and 6 ethanol-exposed animals. In order to assess the effect of early alcohol exposure on the visual system we first examined the retinal responses of ethanol and saline control animals at P30. Using a dark adapted ERG we were able to record the response of retinal cells to a flash of light. This response can be divided into two waves, as shown in **Figure 3A**. First, a fast downward deflection called a-wave is seen, which represents the hyperpolarizing responses of rod and cone photoreceptors to the light flash. The a-wave is followed by a slower upward deflection called b-wave, which represents the light-induced depolarization of ON-bipolar cells (28). A change in these currents could indicate a problem in phototransduction or synaptic transmission between photoreceptors and bipolar cells. Animals were dark adapted overnight, and ERGs were recorded in both eyes. A t-test showed that there was a difference in amplitude for both a-wave and b-wave responses between ethanol and saline groups. Animals treated with ethanol displayed a significantly decreased a-wave amplitude (117 ± 14.5 mV) compared to their saline-treated littermates (216 ± 22.5 mV; $t = 2.1$; $p < 0.01$). This decrease in response was also seen in b-wave amplitudes with ethanol-treated responses again being significantly smaller than controls (Ethanol $= 249 \pm 33.4$ mV; Saline $= 454 \pm 49.2$ mV; $t = 2.1$, $p < 0.01$) (**Figure 3B**). We found no significant difference between the a-wave/b-wave mean ratio of ethanol-treated animals (0.48 ± 0.01) compared to those treated with saline (0.48 ± 0.01; $t = 0.3$, df $= 16$, $p = 0.7$). This finding supports a more direct effect of alcohol on photoreceptors rather than an alteration of the synaptic transmission between photoreceptors and bipolar cells.

OPTICAL IMAGING OF INTRINSIC SIGNALS

We investigated the effect of alcohol on the functional retinotopic organization of the primary visual cortex using an optical imaging technique of intrinsic signal mapping. A drifting white bar moving vertically or horizontally was used as the visual stimulus to obtain elevation or azimuth retinotopic maps, respectively. **Figure 4A** shows representative maps of ethanol and saline-treated animals. Three measurements were used to examine retinotopy – magnification factor, phase scatter, and map tilt.

Magnification factor (degree per millimeter) is defined as the degrees of space in the visual field represented per unit distance in the visual cortex. A lower magnification factor would mean a bigger representation of the visual field in an area of the visual cortex. For elevation, mice treated with ethanol did not show significantly different magnification factors compared to saline (Saline: 44.8 ± 2.2°/mm; $n = 7$ versus Ethanol: 36.0 ± 2.0°/mm; $n = 7$; $t = 1.13$, df $= 12$, $p = 0.28$). Similarly for azimuth, we found no significant change in magnification factors of ethanol-treated animals compared to saline (Saline: 45.6 ± 2.1°/mm; $n = 7$ versus Ethanol: 56.2 ± 6.7°/mm; $n = 4$; $t = -0.86$, df $= 9$, $p = 0.4$) (**Figure 4B**).

Scattering measures how well-defined phase bands are and high scatter is indicative of a poor quality map. Each phase in a retinotopic map (assigned to different colors) represents the region of the visual cortex that responds to the drifting white stimulus bar when it is in a particular location in the animal's visual field. Note that the maps presented show clear separation between phases in both elevation and azimuth maps (**Figure 4A**). Therefore, lower phase scatter values would correspond to smooth progression across phases and a well-organized cortical retinotopy. To calculate phase scatter, we measured the difference (in units of standard deviation) between the phase value of individual pixels within the responsive visual cortex and the mean

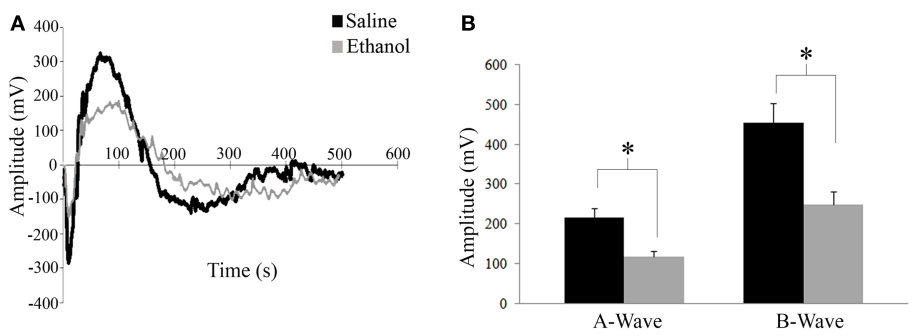

FIGURE 3 | Electroretinography. **(A)** Retinal responses of saline and ethanol-treated animals. First a fast hyperpolarizing a-wave is seen, which represents the response of rod and cone photoreceptors to the light flash. This is followed by the positive b-wave, which represents depolarizing bipolar cell currents. **(B)** ERG recordings after dark adaptation show a difference in amplitude for both a-wave and b-wave responses between ethanol and saline groups. A-wave amplitudes (ethanol: 117 ± 14.5 mV versus saline: 216 ± 22.5 mV; $t = 2.1$; $p < 0.01$). B-wave amplitudes (ethanol: 249 ± 33.4 mV; saline: 454 ± 49.2 mV; $t = 2.1$, $p < 0.01$).

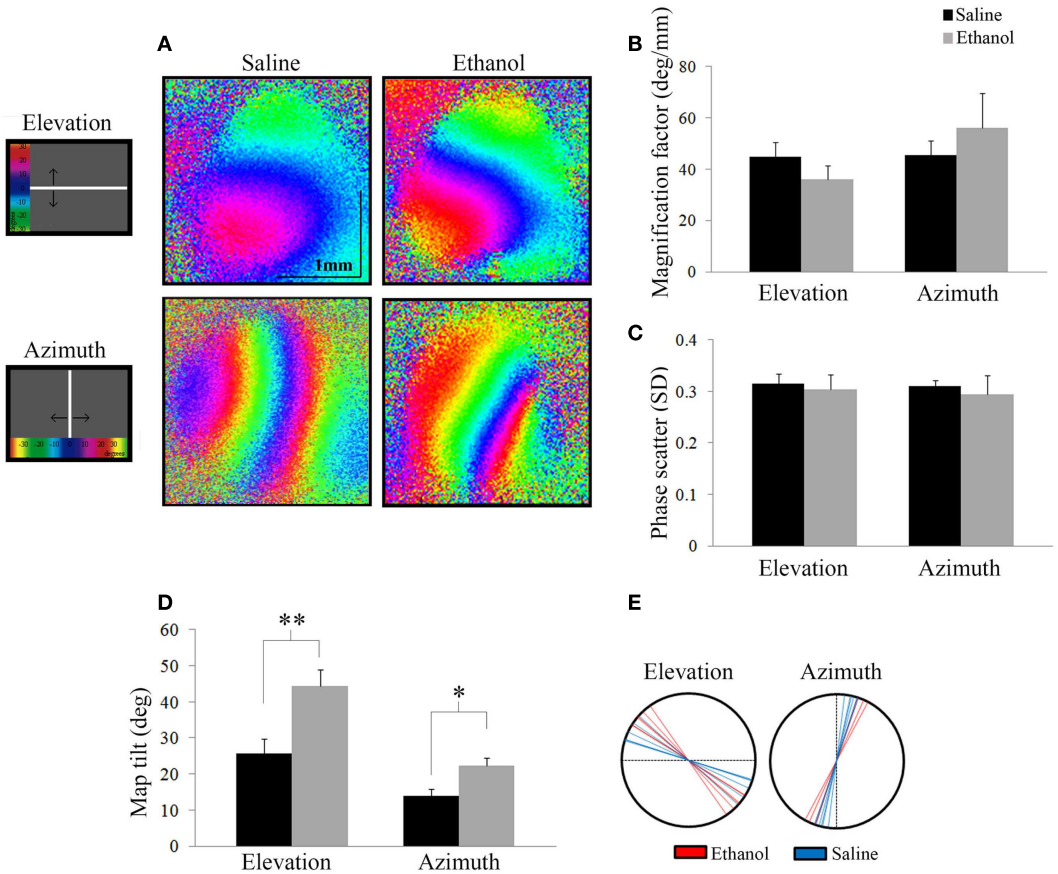

FIGURE 4 | Optical imaging of intrinsic signals. **(A)** Representative elevation and azimuth retinotopic maps from different saline and ethanol-treated animals. To the left of the maps is a schematic of the corresponding visual stimuli presented, with a color scale representing degrees of the visual field. **(B)** Magnification factors of ethanol-treated animals were no different from that of saline-treated animals for elevation ($t = 1.13$, df $= 12$, $p = 0.28$) and azimuth ($t = -0.86$, df $= 9$, $p = 0.4$), signifying that the representation of the visual field in the cortex is similar between the two groups. **(C)** Phase scatter of cortical retinotopy shows no significant difference between saline and ethanol-treated animals in either elevation ($t = 0.1$, df $= 12$, $p = 0.9$) or azimuth ($t = 0.3$, df $= 9$, $p = 0.8$) maps. **(D)** The mean angles of map tilt were significantly different between ethanol and saline-treated animals, for both elevation (ethanol: $44.4 \pm 4.5°$, $n = 7$ versus saline: $25.7 \pm 4.2°$, $n = 6$; $t = -3.02$, df $= 11$, $^{**}p = 0.01$) and azimuth maps (ethanol: $22.3 \pm 2.3°$, $n = 4$ versus saline: $14.0 \pm 1.8°$, $n = 6$; $t = -2.85$, df $= 8$, $^{*}p < 0.05$). **(E)** Each line represents individual values (degrees) of map tilt in ethanol and saline-treated animals for both elevation and azimuth maps.

phase value of their neighboring pixels. There was no significant difference between the phase scatter in the elevation maps of ethanol-exposed animals (0.30 ± 0.03; $n = 7$) compared to saline (0.32 ± 0.02; $n = 7$; $t = 0.1$, df $= 12$, $p = 0.9$), nor in the azimuth maps of animals exposed to ethanol (0.29 ± 0.04; $n = 4$) compared to control (0.31 ± 0.01; $n = 7$; $t = 0.3$, df $= 9$, $p = 0.8$) (**Figure 4C**).

Finally, we investigated a possible effect of ethanol exposure on the orientation of primary visual cortex (V1) retinotopy by measuring the angle of the 0° phase band with respect to the X or Y axis, for elevation and azimuth maps, respectively. We found that this "map tilt" in elevation maps was almost twice that of control, with seven ethanol-exposed mice displaying a mean angle of $44.4 \pm 4.5°$ compared to six saline animals that had a mean angle of $25.7 \pm 4.2°$ ($t = -3.02$, df $= 11$, $p = 0.01$). Similarly, the azimuth maps of ethanol-exposed mice were significantly rotated compared to saline (Ethanol: $22.3 \pm 2.3°$, $n = 4$ versus Saline: $14.0 \pm 1.8°$, $n = 6$; $t = -2.85$, df $= 8$, $p < 0.05$) (**Figures 4D,E**).

DISCUSSION

Our findings demonstrate altered visual properties in adolescent animals previously exposed to ethanol during the third trimester equivalent of human gestation. We first demonstrated a decrease in VEP amplitudes in alcohol-treated animals when compared to controls, which might be a consequence of weak responses of individual neurons or due to a reduced number of cells. The latter seems to be more likely due to the dramatic effect of alcohol exposure in triggering neuroapoptosis, especially when the exposure is during the first 2 weeks after birth (22). Moreover, previous studies from our lab showed that early alcohol exposure does not change visual responsiveness of individual neurons in the primary visual cortex of the ferret (29, 30). Another possible cause of suppressed VEP amplitude could be a loss of feed-forward visual drive, due to a decrease in the myelinated fraction of axons within the optic nerve of animals exposed to a high dose of ethanol during the third trimester equivalent (31). This type of alcohol exposure also results

in a decrease in cell number in the retinal ganglion layer of the retina and the dorsolateral geniculate nucleus (21, 32, 33). Additionally, the remaining retinal ganglion cells (RGCs) demonstrate reduced soma size, and dendritic length (32). The changes in RGC number and their properties may also contribute to the decrease in contrast sensitivity, as seen in VEPs, and the decrease in retinal response, as seen in ERG. Indeed, animals previously exposed to ethanol have been shown to have dose-dependent changes in rhodopsin, resulting in less visual input to the retina (16). Despite these changes, there did not appear to be an effect of early alcohol exposure on spatial frequency acuity.

In late gestation models of FASD, there are no gross ocular malformations as seen in first trimester equivalent ethanol exposure in animals and humans (34). In contrast, a single dose of ethanol on embryonic day 7 results in gross ocular malformations including abnormal lens development, defects of the cornea, and abnormal formation of the anterior chamber (6). Additionally, ethanol exposure throughout the first and second trimester results in a disruption of bipolar and horizontal cells of the retina, resulting in irreversible delays in retina development (35). These early retinal effects are responsible for many of the visual deficits seen in children with FASD, such as changes in acuity due to refractive error, and over-all poor visual function (1, 36). Interestingly, although our animals exhibit no gross eye malformations, we demonstrated an alteration in the receptive fields of V1.

Some of the most important developmental benchmarks of the visual system in rodents occur during the third trimester equivalent of human gestation. It is during this time that the retina fires Stage I, II, and III retinal waves, which are responsible for establishing the organization, binocularity, and connectivity of the structures within the visual system (37–39). The establishment of receptive fields in both V1 and the superior colliculus (SC) are strongly influenced by these retinal waves, as they are propagated simultaneously through the visual system (40). During our ethanol exposure paradigm, Stage III retinal waves are occurring. These waves are predominantly mediated by nicotinic acetylcholine receptors (nAChR), and have been shown to be required (along with correlated RGC activity) for the refinement of retinotopic maps (17, 37). Not surprisingly, ethanol modulates nAChR currents (41). This modulation of retinal wave currents could be responsible for our observed changes in V1 retinotopy.

In addition to retinal waves, ephrin gradients also help to establish retinotopic maps in both the SC and V1 (42). Early alcohol exposure between P4 and P7 has been shown to disrupt ephrin signaling pathways, which support the observed disruption in V1 retinotopic maps (43). As V1 and the SC share mechanisms underlying the establishment of their respective retinotopic maps (i.e., ephrin gradients, retinal waves), it would be interesting to test whether our model of alcohol exposure can also disrupt the retinotopy in the SC. Interestingly, the SC plays a major role in saccades, a visual process that is strongly affected in children with FASD (9, 11).

In conclusion, our findings show in a mouse model that alcohol exposure, restricted to the equivalent of the last months of human gestation, leads to clear visual processing deficits. These findings suggest that tests to assess visual function may be able to contribute to the diagnosis of prenatal alcohol exposure, especially when major craniofacial dysmorphologies are not present. Moreover, our results support the notion that FASD subjects can present visual problems even in absence of spatial frequency acuity deficits. It is conceivable that reduced contrast sensitivity, together with other visual problems, may aggravate learning problems observed in FASD children at school age.

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Analysis of preplate splitting and early cortical development illuminates the biology of neurological disease

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The development of the layered cerebral cortex starts with a process called preplate splitting. Preplate splitting involves the establishment of prospective cortical layer 6 (L6) neurons within a plexus of pioneer neurons called the preplate. The forming layer 6 splits the preplate into a superficial layer of pioneer neurons called the marginal zone and a deeper layer of pioneer neurons called the subplate. Disruptions of this early developmental event by toxin exposure or mutation are associated with neurological disease including severe intellectual disability. This review explores recent findings that reveal the dynamism of gene expression and morphological differentiation during this early developmental period. Over 1000 genes show expression increases of ≥2-fold during this period in differentiating mouse L6 neurons. Surprisingly, 88% of previously identified non-syndromic intellectual-disability (NS-ID) genes are expressed at this time and show an average expression increase of 1.6-fold in these differentiating L6 neurons. This changing genetic program must, in part, support the dramatic cellular reorganizations that occur during preplate splitting. While different models have been proposed for the formation of a layer of L6 cortical neurons within the preplate, original histological studies and more recent work exploiting transgenic mice suggest that the process is largely driven by the coordinated polarization and coalescence of L6 neurons rather than by cellular translocation or migration. The observation that genes associated with forms of NS-ID are expressed during very early cortical development raises the possibility of studying the relevant biological events at a time point when the cortex is small, contains relatively few cell types, and few functional circuits. This review then outlines how explant models may prove particularly useful in studying the consequence of toxin and mutation on the etiology of some forms of NS-ID.

Keywords: preplate, reelin, fetal alcohol spectrum disorders, dendritogenesis, Golgi apparatus

INTRODUCTION

Neocortical development in human is initiated in the seventh week of gestation by the appearance of a layer of pioneer neurons, called the preplate or primordial plexiform layer (1, 2). Preplate neurons lie underneath the meninges and ultimately these neurons cover both cerebral vesicles. At this time, the human cortical wall is only ~250 μm thick, the majority of which is ventricular zone (VZ) (neural precursor cells) and the remaining 20–30 μm contains these early differentiating neurons of the preplate (3, 4). For comparison, the mouse preplate stage corresponds to embryonic day 12.5 post conception (E12.5), a time when the cortical wall has a similar composition of cells and a similar thickness (~150 μm) to the human preplate stage cortex.

Starting at the seventh to eighth week of gestation in human or E13.5 in mouse, a process called preplate splitting initiates the formation of cortical layering. In the mouse, preplate splitting begins in the lateral neocortex and proceeds dorsally and caudally over the next embryonic day (5). Preplate splitting is an early event in cortical development and involves the establishment of an organized layer of cortical plate (CP) neurons within the preplate. The establishment of future layer 6 (L6) neurons splits the preplate into a superficial layer of pioneer neurons called the marginal zone (MZ) and a deeper layer of pioneer neurons called the subplate (SP) (6–10). Preplate splitting is the first step in the formation of the layered cortex and is followed by the successive migration and lamination of cortical layers 5–2 in an inside out fashion (11).

PIONEER NEURONS OF THE PREPLATE

Both MZ neurons and SP neurons have essential roles in organizing the developing cortex (12, 13). MZ neurons, primarily Cajal–Retzius cells, secrete a critical chemotropic factor called Reelin (14) (discussed below) that is required for correct positioning of migrating CP neurons. SP neurons constitute a diverse group of cells (15, 16), which are essential for correct thalamocortical afferent targeting (17–19). Absent correct preplate splitting, the SP cells remain superficial in the cortex and both cortical layering (20) and thalamocortical targeting is disrupted (21, 22). Thus, preplate splitting is a fundamental event that enables the later assembly of the upper cortical layers and leads to a properly formed cerebral cortex (23–25).

GENE EXPRESSION DURING EARLY CORTICAL DEVELOPMENT

The significance of preplate splitting is underscored by the large number of genes specifically upregulated during this period. A prior study used fluorescence activated cell sorting to purify genetically labeled, developing L6 neurons (26). Sorted cells from the transgenic Eomes:eGFP[1] mouse embryos (27) were subjected to RNA extraction and Affymetrix gene chip analysis. In these embryos, enhanced green fluorescent protein (eGFP) expression is under the control of the Eomes (Tbr2) promoter. Eomes is a transcription factor that is selectively expressed by intermediate neural precursor cells of the glutamatergic cortical lineage (28, 29). In transgenic embryos, GFP expression is transient but persists for several days in immature post mitotic neurons of the excitatory cortical lineage. By comparing the GFP^+ population, primarily immature neurons, to the GFP-population, primarily neural precursors, up and down regulated genes in the differentiating excitatory cortical lineage were identified. Approximately half the genome was expressed by these neurons and more than 1000 genes show expression increases $\geq$2-fold during the first ~24–36 h after cell cycle exit (26). Genes of interest could then be validated by comparison to the Genepaint *in situ* database[2]. This prior study validated, and grouped by spatial expression pattern, 317 genes that were upregulated $\geq$3-fold during early cortical neuron differentiation. Importantly, over half of these highly upregulated genes have been associated with neuronal disease (26).

This dataset is a valuable resource that can be queried for genes specifically linked to neurological disorders including nonsyndromic intellectual disability (NS-ID) (30). Of 46 human NS-ID genes identified previously (30), 43 were represented within this dataset, i.e., represented on the mouse Affymetrix Gene 1.0 ST Array (**Table 1**). Of these 43, 38 are expressed above a stringent threshold of RMA = 7.0, and these genes display an average expression level of RMA = 9.5, placing them in the approximate top third of all expressed genes in these immature neurons. At the onset of preplate splitting (E13.5), the expressed genes display an average increased expression of 1.6-fold in GFP^+ neurons versus GFP-precursors. Surprisingly, only 2 genes of the 38 (MAGT and ARX) were downregulated $\geq$1.5-fold in differentiating neurons (i.e., more highly expressed in neural precursors than in differentiating neurons). Thus, the majority of identified NS-ID genes are highly expressed and upregulated by differentiating CP neurons during this early differentiation period, well prior to synapse formation.

What functions might these NS-ID genes be performing during this early period? Expression analysis identified 15 out of 38 (40%) of these early expressed NS-ID gene products as being localized to the nucleus with most of these genes having functions in transcription, chromosomal remodeling, or RNA transport (**Table 1**) (30). An additional 10 of 38 (26%) of the predicted gene products localize to the plasma membrane, where they perform diverse functions as synaptic proteins, adhesion proteins, transporters, and receptors. This group includes Cadherin15 (CDH15) and a subunit of an ionotropic glutamate receptor (GRIK2). Four out of five NS-ID gene products that localize to the cytoplasm are involved in the regulation of small GTPases, namely, Rho, Cdc42, and Rab1, which coordinate cytoskeletal remodeling and vesicular transport, respectively. The five most upregulated NS-ID genes (STXBP1, SYP, FMR2, KIRREL3, and CASK) encode proteins with likely synaptic function. This was a surprise, since there are no morphologically identified synapses on L6 neurons at this time in development. While some of these early expressed mRNAs may not encode functional proteins, it is likely that many NS-ID genes have important roles during this very early period of cortical development.

CELLULAR DYNAMICS DURING EARLY CORTICAL DEVELOPMENT

This dynamic transcriptional profile may underlie the coincident processes of cortical neuron migration and molecular differentiation. Before achieving their mature form, cortical neurons are known to transition through multiple morphological states: from multipolar neuron to radial glial-associated migrating neuron to post migratory differentiating neuron (31–34). Immediately after cell cycle exit, the immature neuron adopts a multipolar morphology and migrates slowly through intermediate zone (IZ) (33, 34) while simultaneously initiating an axon (35, 36). The multipolar neuron, trailing an axon, continues migration until it reaches the SP, the layer of pioneer neurons that underlie the forming CP. At the SP, migrating neurons change from the multipolar shape to a bipolar shape coincident with their attachment to a radial glial fiber (37). The neuron, now apposed to the radial glial fiber, migrates through the developing CP in a saltatory (stepwise) fashion (38). As the neuron approaches the top of the CP, the neuron detaches from the radial glial fiber and translocates into position underneath the MZ (future layer 1) (38) where it elaborates an apical dendrite and becomes excitable.

The sequence of morphological changes is less understood during the earlier period of preplate splitting. Two models have been proposed to account for the appearance of L6 neurons within the preplate. The first model posits direct somal translocation of the immature neuron from the VZ into the preplate (38, 39). This translocation involves the rapid movement of the nucleus into the leading process of the neuron and is thought to occur independent of radial glial guidance or attachment (38). Thus, as more L6 neurons translocate into the preplate, the preplate is split into the MZ and SP. This model, however, appears inconsistent with prior histological observations using electron microscopy (7, 40) and the Golgi stain method (7, 40), or with more recent observations made from the Eomes:eGFP embryonic cortex (10). In these animals, GFP expressing neurons of the excitatory lineage were found intermixed with Calretinin expressing preplate neurons, *prior* to preplate splitting (**Figures 1A–C**). Furthermore, below this mixture of preplate and L6 neurons lies a thick IZ composed of multipolar neurons that do not show translocating morphology (i.e., highly elongated in the radial direction). Thus, the GFP^+ cells that are poised to enter the developing CP are not translocating from the VZ. Instead, preplate splitting appears to be initiated by polarized dendritic growth of L6 neurons and the concurrent coalescing of these L6 neurons into an organized and recognizable CP.

[1] http://www.gensat.org
[2] http://www.genepaint.org

Table 1 | List of non-syndromic intellectual disability (NS-ID) genes expressed in immature excitatory neurons.

Gene	Affymetrix ID	Non-syndromic/ syndromic	Gene name	Gene function	Protein localization	E13.5 GFP^- precursor RMA	E13.5 GFP^+ neuron RMA	E14.5 GFP^+ neuron RMA	E13.5 RMA fold-up	E14.5 RMA fold-up
ACSL4	10607089	NS	Acyl-CoA synthetase long-chain family member 4	Fatty acid metabolism	Mito	8.3	8.5	8.0	1.2	0.8
AFF2/FMR2	10599927	NS	Fragile X mental retardation 2	DNA binding protein/activator of transcription?	Nuc	8.0	9.9	9.7	3.7	3.2
AGTR2	10599001	NS/S	Angiotensin II receptor, type 2	G-protein-coupled receptor/programed cell death	PM	5.4	5.3	5.5	0.9	1.0
AP1S2	10603051	NS/S	Adaptor-related protein complex 1 sigma 2 subunit	Clathrin recruitment and sorting/synaptic vesicles	Golgi	8.8	9.3	9.2	1.4	1.3
ARHGEF6	10604713	NS	Rac/Cdc42 guanine nucleotide exchange factor 6	GEF for Rac and Cdc42	Cyto	6.1	4.5	4.7	0.3	0.4
ARX	10600755	NS/S	Aristaless related homeobox	Transcriptional regulation during development	Nuc	9.8	7.2	7.4	0.2	0.2
ATRX	10606263	NS/S	Transcriptional regulator ATRX	Chromatin remodeling	Nuc	10.2	10.5	10.4	1.2	1.2
BRWD3	10606393	NS/S	Bromo domain and WD repeat protein 3	JAK/STAT signaling in drosophila/chromatin modifier?	Nuc	8.7	9.3	9.0	1.5	1.2
CASK	10603708	NS/S	Calcium/calmodulin-dependent serine kinase	Kinase and scaffolding at synapses/MAGUK family protein	Syn, PM, Nuc, Cyto	10.3	11.5	11.4	2.2	2.2
CC2D1A	10580100	NS	Coiled-coil and C2 domain containing 1A	Transcriptional regulator/NF-κB pathway activator	Nuc, Cyto	7.6	7.9	7.9	1.2	1.2
CDH15	10576175	NS/S	Cadherin 15	Intercellular adhesion protein	PM	7.1	7.0	7.1	0.9	1.0
CRBN	10546775	NS	Cereblon	Expression of potassium channels	PM, Cyto	9.4	10.0	9.8	1.5	1.3
DLG3	10601062	NS	Synapse-associated protein 102	Post-synaptic density scaffold/MAGUK family protein	Syn, PM, ER, Cyto	8.9	9.5	9.5	1.5	1.5
DOCK8	10462140		Dedicator of cytokinesis 8	GEF?/F-actin organization	PM, Cyto, Nuc	6.1	6.1	6.1	1.0	1.0
FGD1	10602401	NS/S	Faciogenital dysplasia protein	GEF for Cdc42	Cyto	9.3	9.1	9.2	0.9	0.9
FTSJ1	10603508	NS	FtsJ homolog 1	rRNA processing	Nuc	9.8	9.9	9.7	1.1	0.9

(Continued)

Table 1 | Continued

Gene	Affymetrix ID	Non-syndromic/ syndromic	Gene name	Gene function	Protein localization	E13.5 GFP^- precursor RMA	E13.5 GFP^+ neuron RMA	E14.5 GFP^+ neuron RMA	E13.5 RMA fold-up	E14.5 RMA fold-up
GDI1	10600390	NS	GDP dissociation inhibitor 1	Inhibitor of Rab GTPases	Cyto	11.7	12.8	12.5	2.2	1.8
GRIK2	10368999	NS	Glutamate receptor, ionotropic,, kainate 2	Subunit of glutamate receptor (kainate)	PM, Syn	9.7	10.2	9.2	1.4	0.7
HUWE1	10602501	NS/S	HECT, UBA, and WWE domain containing 1	Ubiquitin E3 ligase/protein ubiquitination	Nuc, Cyto	10.5	10.5	10.6	1.0	1.0
IL1RAPL1	**NA**	NS	Interleukin 1 receptor accessory protein-like 1	Vesicle release/dendrite differentiation	PM	**NA**				
JARID1C/ KDM5C	10602644	NS	Jumonji, AT rich interactive domain 1C	Transcriptional regulation/chromatin remodeling	Nuc	9.9	9.8	10.0	0.9	1.1
KIRREL3	10584165	NS/S	Kin of IRRE like 3	Synaptogenesis?	PM, Cyto, EC	7.5	8.9	9.3	2.5	3.3
MAGT1	10606301	NS	Magnesium transporter 1	Mg^{2+} uptake/N-glycosylation	ER	10.4	8.5	8.2	0.3	0.2
MBD5	10471967	NS/S	Methyl-CpG binding domain protein 5	Transcriptional regulation?	Nuc	7.8	8.5	8.5	1.6	1.6
MECP2	10605247	NS/S	Methyl-CpG binding protein 2	Transcriptional regulation	Nuc	8.8	9.0	9.1	1.1	1.2
NLGN4X	10601152	NS	X-linked neuroligin 4	Synaptic adhesion protein	PM, Syn	9.5	9.9	9.8	1.3	1.2
OPHN1	10605884	NS/S	Oligophrenin 1	Rho-GTPase activating protein	Cyto	8.2	9.3	9.3	2.2	2.1
PAK3	10602198	NS	p21-activated kinase 3	Effector of Rho-GTPases	Cyto	9.9	10.1	9.9	1.1	1.0
PQBP1	10603373	NS/S	Polyglutamine binding protein 1	Transcriptional regulation	Nuc, Cyto	10.9	11.3	10.8	1.3	1.0
PRSS12	10495854	NS	Neurotrypsin	Synaptic protease/cleaves agrin/synaptic plasticity	EC	6.9	7.3	7.1	1.3	1.2
PTCHD1	10607486	NS	Patched domain 1	Hedgehog receptor?	PM	6.4	6.5	6.4	1.1	0.9
RPS6KA3	10602772	NS/S	Ribosomal protein S6 kinase, 90kDa, polypeptide 3	Ras/Map/ERK regulation	Cyto	9.8	9.8	9.6	1.0	0.9
SHANK2	10559343	NS	SH3 and multiple ankyrin repeat domains 2	Scaffolding and cell adhesion protein/synaptic plasticity	Cyto, Syn	7.1	7.7	7.9	1.5	1.7

(Continued)

Table 1 | Continued

Gene	Affymetrix ID	Non-syndromic/ syndromic	Gene name	Gene function	Protein localization	E13.5 GFP^- precursor RMA	E13.5 GFP^+ neuron RMA	E14.5 GFP^+ neuron RMA	E13.5 RMA fold-up	E14.5 RMA fold-up
SHROOM4	10598240	NS/S	Shroom family member 4	Cytoskeletal architecture	Cyto	6.2	5.7	5.8	0.7	0.8
SLC6A8	10600210	NS	Solute carrier family 6 member 8	Creatine transporter	PM	8.4	8.3	8.5	0.9	1.1
STXBP1	10481711	NS	Syntaxin-binding protein 1	Synaptic vesicle docking and fusion/neurotransmission	PM, Syn, Cyto	8.6	10.8	10.9	4.6	5.0
SYNGAP1	10443091	NS	Synaptic Ras GTPase activating protein 1	NMDA receptor complex/Ras/Map/ERK regulation	PM, Syn	9.6	10.2	10.7	1.6	2.1
SYP	10598359	NS/S	Synaptophysin	Synaptic vesicle protein	Syn	8.8	10.9	10.6	4.2	3.5
TSPAN7	10598626	NS/S	Tetraspanin 7	Synapse maturation?	PM, Syn	11.9	11.7	11.2	0.8	0.6
TRAPPC9	**NA**	NS	NIK- and IKKB-binding protein	Neuronal NF-κB signaling/vesicular transport	Golgi, ER, Cyto	**NA**				
TUSC3	10571371	NS	Tumor suppressor candidate 3	Mg^{2+} uptake/oligosaccharide transferase/N-glycosylation	ER	9.3	10.2	10.2	1.8	1.8
UPF3B	10604078	NS/S	UPF3 regulator of nonsense transcripts homolog B	mRNA nuclear export and surveillance	Nuc, Cyto	7.9	8.1	8.0	1.1	1.1
ZNF41/ zfp27 56%	**NA**	NS	Zinc finger protein 41	Putative repressor of transcription	Nuc	**NA**				
ZNF674/ zfp182 56%	10603881	NS	Zinc finger protein 674	Putative repressor of transcription	Nuc	6.9	7.5	7.2	1.5	1.2
ZNF711/ zfp711 98%	10601492	NS	Zinc finger protein 711	Activator of transcription?	Nuc	8.6	9.7	9.4	2.1	1.7
ZNF81/ zfp160 47.4%	10442172	NS	Zinc finger protein 81	Repressor of transcription?	Nuc	8.3	8.9	8.6	1.5	1.2

A dataset of genes expressed by immature mouse cortical neurons at E13.5 and E14.5 (26) was queried for the expression of human NS-ID orthologs identified in (30). The expression values are reported as RMA (robust multichip average) as a log2 scale (e.g., RMA 9.0 is twofold higher than RMA 8.0). The fold-up values are derived from comparing the expression of the gene in the GFP^+ neuronal population to the expression of the gene in GFP-neural precursors. Highlighted rows identify genes that are either not represented in the mouse data set (NA) or are expressed at levels below threshold (RMA = 7.0). Mouse zinc finger protein (Zfp) orthologs are listed with their percent amino acid identity to the corresponding human zinc finger protein (ZNF). The human NS-ID table is modified from Kaufman et al. (30) with permission.

EC, extracellular; ER, endoplasmic reticulum; Golgi, Golgi apparatus; Syn, synapse; PM, plasma membrane; Nuc, nucleus; Mito, mitochondria.

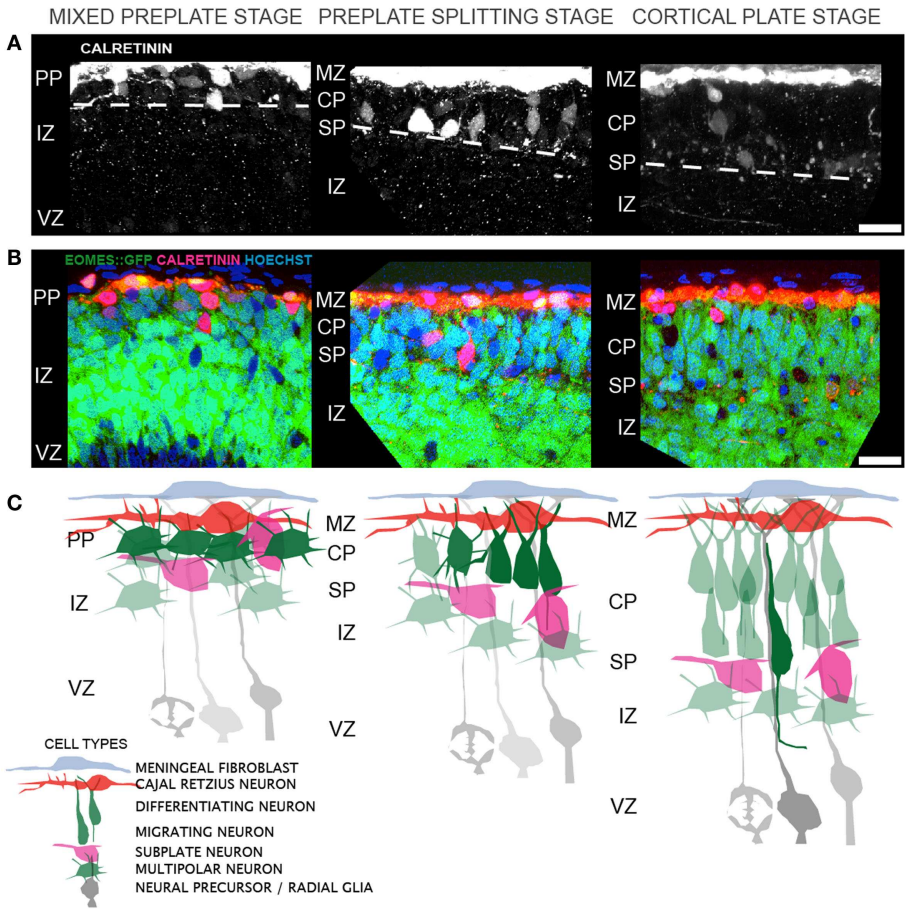

FIGURE 1 | Cellular events that contribute to cortical layer 6 formation and preplate splitting. **(A)** Distribution of Calretinin immunopositive preplate neurons prior to (left), during (middle), and after (right) preplate splitting. **(B)** A single confocal slice through the Eomes:eGFP cortex at the same regions as in (A), showing the distribution of GFP^+ excitatory cortical neurons (green) and $Calretinin^+$ preplate neurons (red) and $Hoechst^+$ nuclei (blue). **(C)** Model of the corresponding cellular rearrangements during the mixed preplate stage, where L6 neurons are intermingled with preplate neurons, the preplate splitting stage, where L6 neurons become radially oriented and coalesce into a recognizable cellular layer and the cortical plate stage, where migrating neurons enter the CP on radial glial fibers. Abbreviations: marginal zone (MZ), preplate (PP), cortical plate (CP), subplate (SP), intermediate zone (IZ), ventricular zone (VZ). Scale bar is 25 μm in **(A,B)**.

The calcium-binding protein Calretinin is a marker for subsets of both MZ and SP neurons during early rodent cortical development (41, 42) and therefore the separation of preplate $Calretinin^+$ cells into the MZ and SP groups is a hallmark of preplate splitting. In this model, $Calretinin^+$ MZ neurons stay in place and $Calretinin^+$ SP neurons either actively migrate away (43) from or are passively displaced by the coalescing L6 neurons. Thus, the initial phase of preplate splitting is driven by active reorganization of these L6 neurons, rather than their translocation. Future imaging studies should help resolve these two models. In both models, however, the period of preplate splitting represents a period of dynamic cellular transformations.

DISRUPTIONS OF EARLY CORTICAL DEVELOPMENT

Disruptions of preplate splitting either by toxin or mutation (44) are associated with serious neurological disability including mental retardation, epilepsy (45), and possibly autism (46). Prenatal exposure to alcohol is a leading cause of mental retardation and intellectual disability (47, 48). The CDC estimates that 0.2–1.5 per 1000 live births are children with fetal alcohol syndrome (FAS), a syndrome defined by mental dysfunction (49). The cognitive deficits caused by prenatal exposure to EtOH are likely reflected in the specific functional and structural abnormalities found in brains of alcohol-exposed children (50, 51).

EtOH exposure is known to impact neuronal plasticity and these disruptions range from the short term (e.g., memory deficits caused by binge drinking) (52–54) to long term [e.g., disruption in memory and cognition associated with alcoholism (55)] to permanent [e.g., structural changes and intellectual disability associated with FASD (56, 57)] or chronic alcoholism (58). The disruptions caused by ethanol exposure vary with time period of exposure (59, 60). This differential sensitivity to ethanol may reflect the major underlying cellular processes occurring at the time of exposure (61).

Although EtOH exposure strongly promotes apoptosis during the synaptic formation period (62), EtOH can also target multiple

events prior to synapse formation including neurogenesis, neuronal migration (63), axonal outgrowth (64, 65), and dendritic development (66–68). These biologically important processes can be assayed using early embryonic cortical explants. At this time, the cortex is small, composed of relatively few cell types and have few synapses. Nevertheless, these explants captures critical organotypic interactions including signals derived from other neurons as well as non-neuronal elements including radial glia (69, 70), blood vessels (71), meninges (72), and associated extracellular matrix (14). This organotypic environment provides the multiple substrates and signals that allow cortical neurons to mature through intermediate stages and to finally adopt appropriate form and function. Understanding how EtOH disrupts these signaling systems may be required for a fuller picture of the etiology FASD and the development of NS-ID.

WHOLE HEMISPHERE EXPLANTS

A whole hemisphere explant procedure that permits 2 days of organotypic growth and encompasses the period of preplate splitting has been valuable in understanding the cellular transformations of preplate splitting (10, 73). In this procedure, entire embryonic cortices are isolated with the meninges intact and are then cultured on collagen filters as is done with slice explants (74, 75). Keeping the meninges intact helps preserve the organization of the basal lamina, the radial glial endfeet as well as the pioneer neurons that are found in the MZ. Disruptions of the meninges during development, through mutation (76, 77) or injury (78) can cause focal heterotopia and disrupt underlying cortical layering. Therefore, keeping the meninges as intact as possible is desirable and allows for continuous growth and lamination of the CP during the *in vitro* period. The CP is organized and shows appropriate expression of the transcription factors Tbr1 and Ctip2. Similarly, the radial glial network is intact evidenced by appropriate expression of the intermediate filament protein Nestin (73).

Using the whole hemisphere explant model, it was found that cellular orientation and apical dendritic growth was disrupted by single dose ethanol exposure, with an increase in primary dendrite number detected within 4 h of exposure (67). This dendritic alteration was accompanied by a morphological compaction of the Golgi apparatus, a key support organelle for the growing dendrite (79), as well as a slower reduction in cytoskeletal F-actin and the microtubule associated protein MAP2 content (67). These disruptions are remarkably similar to, but less severe than, disruptions caused by disruption of the Reelin-signaling pathway (80). Reelin is a large glycoprotein that is secreted by Cajal–Retzius cells in the MZ, during the period of preplate splitting (14, 81). Without Reelin, the preplate fails to split (9, 74, 75) and the subsequently generated cortical layers pile up underneath L6 leading to an inversion of cortical layering (20). In human beings, Reelin deficiency leads to mild epilepsy and severe mental retardation (45). At the cellular level in Reelin-deficient (*reeler*) cortical explants, neurons were tangentially oriented rather than radially oriented. The dendritic arbor was simplified and these neurons displayed more primary processes and a compact Golgi apparatus (10, 80). In addition dendritic expression of F-actin and MAP2 was reduced in *reeler* mutants compared to wild-type controls (10). Thus, studies using early cortical explants are showing a potential convergence of cellular phenotypes underlying two etiologically distinct forms of intellectual disability.

CONCLUSION

Early cortical development is a period of remarkable dynamism with large scale changes in the pattern of gene expression, significant tissue growth and a surprising amount of neuronal differentiation. Disruption of early cortical development by exposure to toxin (e.g., EtOH) or mutation (e.g., Reln) can lead to intellectual disability. Explant models of early cortical development provide a bridge between dissociated culture studies and *in vivo* studies. The relatively small size and simple cellular composition allow for the study of disease relevant biology in the absence of synapses and functional circuits.

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Glia and neurodevelopment: focus on fetal alcohol spectrum disorders

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During the last 20 years, new and exciting roles for glial cells in brain development have been described. Moreover, several recent studies implicated glial cells in the pathogenesis of neurodevelopmental disorders including Down syndrome, Fragile X syndrome, Rett Syndrome, Autism Spectrum Disorders, and Fetal Alcohol Spectrum Disorders (FASD). Abnormalities in glial cell development and proliferation and increased glial cell apoptosis contribute to the adverse effects of ethanol on the developing brain and it is becoming apparent that the effects of fetal alcohol are due, at least in part, to effects on glial cells affecting their ability to modulate neuronal development and function. The three major classes of glial cells, astrocytes, oligodendrocytes, and microglia as well as their precursors are affected by ethanol during brain development. Alterations in glial cell functions by ethanol dramatically affect neuronal development, survival, and function and ultimately impair the development of the proper brain architecture and connectivity. For instance, ethanol inhibits astrocyte-mediated neuritogenesis and oligodendrocyte development, survival and myelination; furthermore, ethanol induces microglia activation and oxidative stress leading to the exacerbation of ethanol-induced neuronal cell death. This review article describes the most significant recent findings pertaining the effects of ethanol on glial cells and their significance in the pathophysiology of FASD and other neurodevelopmental disorders.

Keywords: glia, astrocytes, oligodendrocytes, microglia, fetal alcohol spectrum disorders, neurodevelopment

INTRODUCTION

Glial cells were first described by Rudolf Virchow in the middle of the nineteenth century and had been considered until recently as merely supportive and passive elements of the brain. In the last 20 years, there is accumulating evidence that rather than glial cells being supporting players in brain function, they are co-stars with neurons as new and exciting roles for them in brain development, function, and disease have emerged (1).

Neurons and macroglia (which include astrocytes, oligodendrocytes, and ependymal cells) differentiate from common precursor cells, namely neuroepithelial cells that line the cerebral ventricles and spinal canal during early brain development. Some neuroepithelial cells directly differentiate into neurons, although the majority of them are transformed into radial glial cells, which, passing through the stages of intermediate cell-type specific progenitor and precursor cells, generate neurons, oligodendrocytes, and astrocytes (2). Cell-intrinsic and -extrinsic cues regulate the differentiation of neural progenitors into each cell type, enabling the formation of functional neural circuits. Epigenetic mechanisms of chromatin remodeling appear to play a role in the cell fate specification of precursor brain cells (3). The mechanisms regulating the timing and the number of neurons and glial cells generated from a common progenitor are not fully understood. Microglia are the resident macrophages of the brain and spinal cord. The current theory regarding microglia origins is that they derive from immature erythromyeloid progenitors that migrate from the yolk sac blood islands before the blood–brain barrier starts developing (4, 5). Embryonic microglia proliferate during late gestation and early postnatal development and colonize the whole central nervous system (CNS) (4).

This review outlines the main roles played by astrocytes, oligodendrocytes, and microglia, during physiological and pathological brain development; furthermore, this review describes in detail the effects of alcohol on glial cells during brain development and highlights how these effects may contribute to the behavioral and structural effects of alcohol as seen in fetal alcohol spectrum disorders (FASD).

Abbreviations: ABCA1, ATP-binding cassette-A1; ABCG1, ATP-binding cassette G1; ADNP, activity-dependent neuroprotective protein; ARSB, arylsulfatase B; ASD, autism spectrum disorders; CNS, central nervous system; ECM, extracellular matrix; FASD, fetal alcohol spectrum disorders; FMR1, fragile X mental retardation 1; IL1-R1, interleukin1-receptor 1; iPSCs, induced pluripotent cells; MBP, myelin basic protein; MeCP-2, methyl-CpG–binding protein 2; OPCs, oligodendrocyte precursor cells; PAI-1, plasminogen activator inhibitor-1; PD, postnatal day; PKC, protein kinase C; PLD, phospholipase D; SPARC, secreted protein acidic and rich in cysteine; SRF, serum response factor; TLR, toll-like receptor; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

ASTROCYTES IN BRAIN DEVELOPMENT

Astrocytes, the most numerous cells in the mammalian brain, have now been fully recognized as key mediators of brain development, function, and plasticity. Astrocytes serve a particularly profound

role in brain development by in large part coordinating neuronal development through targeted release of trophic factors and extracellular matrix (ECM) proteins leading neurite outgrowths, allowing for neuronal survival, and controlling synapse formation and function (6–15). Furthermore, astrocytes express numerous receptors including receptors for neurotransmitters and neuromodulators that allow them to respond to cues deriving from neurons (16). Thus, astrocytes play a major role in the formation of neuronal circuits.

We demonstrated a new mechanism of astrocyte–neuron interaction by which the stimulation of astrocytes with the cholinergic agonist carbachol leads to the activation of a complex signaling involving phospholipase D (PLD), protein kinase C (PKC) ζ and ε, p70S6 kinase, NF-kB pathway, and MAPK (17–24). This results in an increase in neurite outgrowth of hippocampal neurons in astrocyte–neuron co-cultures and hippocampal slices, an effect mediated by M3 muscarinic receptors (8, 25).

Astrocytes secrete several molecules that are implicated in neurite outgrowth and synaptogenesis. We characterized astrocyte secretome by shotgun proteomic and found that most of the proteins secreted by astrocytes are involved in neuronal development and consist of ECM components as well as proteases, and protease inhibitors that modulate the levels of ECM by affecting the rate of its degradation (26). ECM proteins are indeed involved in astrocyte-modulated neuronal development processes, such as neurite outgrowth and synaptogenesis.

In recent years, several astrocyte-secreted molecules involved in the modulation of synaptogenesis have been identified including ECM proteins thrombospondin, hevin, secreted protein acidic and rich in cysteine (SPARC), membrane-anchored glypican, and lipoproteins (7, 27–29). We have reported that the stimulation of neurite outgrowth by carbachol-treated astrocytes is mediated by increased expression of the ECM proteins fibronectin and laminin in these cells and in the medium and by the upregulation of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of the proteolytic degradation of the ECM (8).

Astrocytes also serve various roles in maintaining brain integrity and health. Astrocytes during brain development play a major role in the maturation, function, and maintenance of the blood–brain barrier (30, 31). In addition, together with microglia, they are responsible for immune function of the CNS contributing to the regulation of the neuroinflammatory response (32).

ASTROCYTES AND NEURODEVELOPMENTAL DISORDERS

Recently astrocytes have been involved in the pathophysiology of several neurodevelopmental diseases. Several studies identified astrocyte dysfunction as a cause of altered development in surrounding neurons; therefore, understanding how astrocyte functions are altered in neurodevelopmental disorders is essential for the development of more effective therapeutic strategies.

Between 2009 and 2010, four independent studies were published indicating a role for astrocytes in four neurodevelopmental disorders, namely Rett syndrome, Fragile X syndrome, Fetal Alcohol Spectrum Disorders (FASD), and Down syndrome (33–36). These studies employed similar *in vitro* models of astrocyte–neuron co-cultures to demonstrate that astrocytes derived from methyl-CpG-binding protein 2 (MeCP2)-lacking mice (a model for Rett Syndrome) (33), astrocytes derived form fragile X mental retardation 1 (FMR1) knock-out mice (a model for Fragile X syndrome) (36), astrocytes pre-treated with ethanol (a model for FASD) (35), and astrocytes derived from human Down syndrome fetuses (34) foster an altered neurite and dendritic spine development in co-cultured rodent hippocampal neurons. A common finding of these studies is that, in these neurodevelopmental disorders, astrocyte dysfunction has profound effects on surrounding neurons. A follow-up study carried out in the Rett syndrome animal model showed that re-expression of MeCP2 in astrocytes improved locomotion, anxiety and respiratory patterns, prolonged lifespan, restored normal dendritic morphology, and increased the levels of the synaptic vesicle protein vGlut1 (37). Dendritic defects of FMR1-deficient neurons are significantly rescued when these cells are grown on a monolayer of wild-type rather than FMR1-deficient astrocytes. FMR1-deficient astrocytes, on the other hand, delay dendritic growth and the formation of excitatory synapses (36).

The non-cell-autonomous effects of astrocytes derived from Down syndrome fetuses on dendritic spines were attributed to reduced expression of the ECM protein thrombospondin-1 (34), which is involved in astrocyte-stimulated synapse formation (7). A recent study carried out in astrocytes and neurons differentiated from induced pluripotent cells (iPSCs) derived from Down syndrome patients demonstrated that Down syndrome astrocytes reduce neurogenesis, induce cell death, and fail to promote maturation and synaptogenesis in Down syndrome neurons. In addition, Down syndrome astrocytes display increased glial fibrillary acidic protein (GFAP) and S100B expression and nitric oxide generation, indicating that they are in a reactive state (38). The antibiotic minocycline, which has been reported to have anti-inflammatory and neuroprotective properties, partially corrects the pathological phenotype of Down syndrome astrocytes.

Reduced neuronal development has been also reported in hippocampal pyramidal neurons co-cultured with astrocytes pre-treated with developmental neurotoxicants, such as the organophosphorus insecticide diazinon, its active metabolite diazoxon, and manganese (39, 40). Alexander's disease is a genetic disease caused by gain-of-function point mutations in the GFAP gene. Alexander's disease is an astrogliopathy, a primary disease of astrocytes. Astrocytes are the major producers of GFAP, and Alexander disease astrocytes present Rosenthal fibers accumulation and increased levels of GFAP. Alexander's disease is characterized by macrocephaly, abnormal white matter, and developmental delay (41, 42). The mechanism of astrocyte dysfunction is not fully understood, but the presence of white matter injury suggests effects on oligodendrocytes and myelination. Astrocyte and microglia activation caused by prenatal infections has been associated with reduced number of oligodendrocytes, altered myelination, and schizophrenia (43, 44).

In conclusion, fast-growing evidence indicates that astrocytes play a major role in neurodevelopmental disorders caused by both, genetic mutations and environmental exposures. Thus, astrocytes may be a new target for the development of therapeutic agents to treat these diseases (45, 46).

OLIGODENDROCYTES IN BRAIN DEVELOPMENT

Central nervous system myelin is an extension of the plasma membrane of oligodendrocytes wrapping multiple times around the axon where it forms a compacted sheath. Myelin allows for the process of saltatory conduction of action potentials propagated between nodes of Ranvier increasing the speed and efficiency of nerve conduction and is specific to vertebrates. Myelin, first described by Ehrenberg in 1833, was initially viewed as a static component surrounding the axons. It is now well established that the myelination process involves complex and dynamic cell–cell interactions (47, 48) that can be modified by functional experience (49, 50). In addition, there is evidence that oligodendrocytes and myelin provide trophic support to axons and promote their integrity and survival (51, 52).

Central nervous system myelination occurs late in brain development: in humans, the majority of CNS myelination occurs during the first two decades of life (53), in rodents, during the first two postnatal months. There is now evidence that in both humans and rodents, myelination continues throughout life (54, 55). Oligodendrocytes are the last cells to be generated during development, although oligodendrocyte precursor cells (OPCs) are produced much earlier in development (starting on E12.5 in mice). OPCs are produced in restricted areas and subsequently migrate and populate the entire brain where they later develop into mature oligodendrocytes. Some OPCs are present in the mature brain where they may be responsible for adult myelination (56). The myelination process is largely driven by intrinsic genetic mechanisms, but increasing evidence indicates that a major role is also played by experience-driven plasticity. Several extracellular signals, intracellular pathways, and transcription factors regulating oligodendrocyte differentiation and myelination have been characterized (52).

OLIGODENDROCYTES AND NEURODEVELOPMENTAL DISORDERS

Several types of prenatal insults have been shown to later affect myelination, indicating that effects on the generation or survival of OPCs during fetal development are responsible for altered myelin in the adult brain. Indeed, it has been reported that gestational exposure to stresses, including hypoxia, restraint stress, opioids, vitamin B12 deficiency, and methamphetamine decrease myelination in the offspring postnatal brain (57–61). Prenatal infections associated with astrocyte and microglia activation induce myelin and oligodendrocytes abnormalities and may be linked to schizophrenia (43, 44).

MICROGLIA IN BRAIN DEVELOPMENT

Microglia are resident CNS immune cells that represent approximately 10% of the total brain cell population (62). Microglia are of hematopoietic origin; their main function in the adult brain is to monitor the environment and to respond to infection and injury. Microglia exist in two conformations: surveying microglia, presenting a ramified morphology, and activated microglia, which can assume an ameboid, rod, multinucleated, or epithelioid morphology (62). Microglia respond to almost all types of CNS insult by switching from a surveillance state to one of the activated states that involves changes in cell morphology, gene expression, and function (62). Activated microglia produce many pro-inflammatory mediators, including cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide. Furthermore, microglia can become phagocytic and contribute to the clearance of pathogen infections and toxic cellular debris after injury. While microglia activation is aimed at protecting neurons from infections, it can also trigger extensive and damaging neuroinflammation that contribute to the progression of neurodegenerative diseases (63).

Microglia are present in the amoeboid morphology during embryonic development and transition toward a ramified morphology during early postnatal development in rodents. During CNS development, microglia play a major role in the refinement of brain wiring and synaptic circuits (64–66). Neuronal apoptosis and synaptic pruning are important physiological processes occurring in the developing brain where more than the necessary neurons and synapses are generated. In the immature brain, amoeboid microglia have an active role in phagocytosis of apoptotic neurons, promotion of programmed cell death (67, 68), and pruning of synapses (69). Mechanisms inducing engulfment of synaptic structures and synaptic pruning by microglia include activity-dependent mechanisms (70), fractalkine signaling (69), and modulation of complement protein release by developing synapses (71).

MICROGLIA AND NEURODEVELOPMENTAL DISORDERS

Several lines of evidence implicate microglia in autistic spectrum disorders (ASD) (72). Several markers of neuroinflammation have been reported in the brain of ASD patients. Indeed, high levels of cytokines, which are expressed in the brain by activated microglia (73), are found in the brains of autistic patients (74, 75). Furthermore, microglial activation has also been demonstrated in several brain regions of ASD patients (76–78).

In a mouse model of Rett syndrome, a genetic disease of the ASD spectrum caused by mutations in the MeCP2 protein, microglia display reduced phagocytic activity; the motor abnormalities present in MeCP2 knock-out animals are partially rescued by replacing MeCP2 in microglia (79). MeCP2-null microglial cells release glutamate, delay neuronal development, and trigger neurotoxicity in hippocampal neurons (80).

Epidemiologic studies show that prenatal exposure to infections is associated with increased risk of adult schizophrenia (81). Because microglia are the main player in neuroinflammatory responses, microglia may be implicated in alterations in brain circuits during the development caused by prenatal infections, which may be involved in schizophrenia (43, 44).

FETAL ALCOHOL SPECTRUM DISORDERS

FASD are a heterogeneous group of conditions defined as the physical, behavioral, and learning impairments that occur in the offspring of women who drank alcohol during pregnancy (82, 83). FASD include fetal alcohol syndrome (FAS), partial FAS (pFAS); alcohol-related neurodevelopmental disorders (ARND), and alcohol-related birth defects (ARBD) (84, 85). Neurobehavioral deficits associated with heavy prenatal alcohol exposure include reduced IQ and impairments in several neurodevelopmental domains such as attention, reaction time, visuospatial abilities,

executive functions, fine and gross motor skills, memory, language, and social and adaptive functions (86).

Glia involvement in FASD is suggested by the fact that the brains of individuals with FASD present with abnormal glial migration (87) and hypoplasia of the corpus callosum and anterior commissure, two areas originally formed by neuroglial cells (88). Furthermore, the finding that microencephaly is strongly associated with ethanol exposure during the brain growth spurt (89), a period characterized by rapid glial cell proliferation and maturation, also suggests a potential effect of ethanol on the proliferation, growth, and maturation of glia. In experimental models, the involvement of glial cells in the developmental effects of ethanol has been recognized for more than 20 years. Earlier findings pertaining to glia and FASD have been summarized in an excellent review (90); therefore, the present review will focus mostly on research published during the last 15 years.

ASTROCYTES AND FASD

ASTROCYTE PROLIFERATION IN FASD

Several early studies investigated the effects of ethanol on astrocyte proliferation. The incubation of primary astrocytes in culture with ethanol inhibits astrocyte proliferation induced by serum, M3 muscarinic receptor stimulation, and IGF-1 (91–93). Subsequent studies revealed selectivity in the signaling pathways affected by ethanol. Muscarinic receptor-mediated activation of phospholipase C, subsequent increase in intracellular calcium, and activation of novel PKCε and of mitogen-activated protein kinases are relatively unaffected by ethanol (24, 94). On the other hand, PLD-mediated formation of phosphatidic acid and sequential activation of atypical PKCζ, p70S6 kinase, and of NF-κB are all strongly inhibited by ethanol (17, 20, 22).

Phospholipase D-induced phosphatidic acid formation was identified by two groups as the direct target of ethanol in the inhibition of muscarinic receptor- and serum-stimulated signaling in astrocytes (22, 95). Indeed, ethanol is a competitive substrate for PLD leading to the formation of phosphatidylethanol instead of the physiological second messenger phosphatidic acid (96). Phosphatidic acid activates a myriad of signaling molecules, including RAF, mTOR, p70S6K, and Akt and is involved in several cellular functions, including intracellular trafficking, survival, and proliferation (97, 98) (**Figure 1**). Inhibition of astrocyte proliferation by ethanol is consistent with the reduced number of glial cells found following *in vivo* ethanol exposure (99, 100), and may contribute to ethanol-induced microencephaly (89).

ASTROCYTES AND NEURONAL PLASTICITY IN FASD

Several paradigms of neuronal plasticity are altered by alcohol exposure during brain development in animal models of FASD, as recently summarized in an excellent review article (101). A large body of evidence suggests that structural plasticity is highly affected by *in utero* alcohol exposure. Indeed, specific cortical maps are altered in FASD models (102–104). Furthermore, prenatal and/or neonatal alcohol exposure reduces dendritic branching and dendritic spine density in hippocampal and neocortical pyramidal neurons (105–110).

The importance of astrocytes in ethanol-induced changes in neuritogenesis is indicated by co-culture experiments. Medium

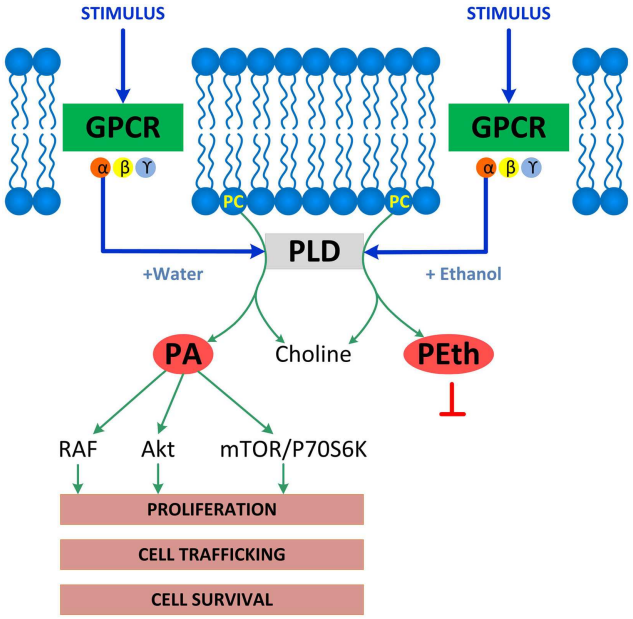


FIGURE 1 | Enzymatic reactions catalyzed by PLD. PLD is associated with membrane receptors including G-protein coupled receptors (GPCR), receptor tyrosine kinases, or integrins, which all activate PLD. Shown is GPCR-coupled PLD, which, upon activation under physiological conditions, hydrolyzes phosphatidylcholine (PC) to produce choline and phosphatidic acid (PA), a lipid second messenger that binds and activates several signaling molecules including RAF, Akt, mTOR, and p70S6K and stimulates several cell functions including proliferation, cell trafficking, and cell survival. The PLD signaling pathway is disrupted by ethanol, which competes with water leading to the formation of phosphatidylethanol (PEth) at the expenses of phosphatidic acid, therefore, inhibiting phosphatidic acid-activated signaling and functions.

from ethanol-cultured astrocytes was shown to impair neuronal survival and neuritogenesis of rhombencephalic serotoninergic neurons in culture (111). Astrocytes have also been reported to modulate the effect of ethanol on dendritic development with different outcomes depending on the time of ethanol exposure (112). Further, neuritogenesis is inhibited in cortical neurons grown in the presence of astrocytes prepared from rats prenatally exposed to ethanol in comparison to neurons incubated with astrocytes from unexposed animals (113).

Several factors released by astrocytes have been implicated in the effects of ethanol on neuritogenesis. The active fragment of the astrocyte-released activity-dependent neuroprotective protein (ADNP) has been implicated in ethanol's effects on axonal growth in cerebellar neurons (114). Serum response factor (SRF) in astrocytes has also been suggested to play a role in the effects of ethanol on neuritogenesis. Indeed, overexpression of SRF in astrocytes restores ocular dominance plasticity in a ferret model of FASD (115, 116).

We have reported on the important role of muscarinic receptors in ethanol-induced impairment in neuritogenesis related to FASD. Hippocampal neuron neuritogenesis stimulated by astrocyte muscarinic receptor is inhibited by physiologically relevant concentrations of ethanol in an astrocyte–neuron co-culture model in

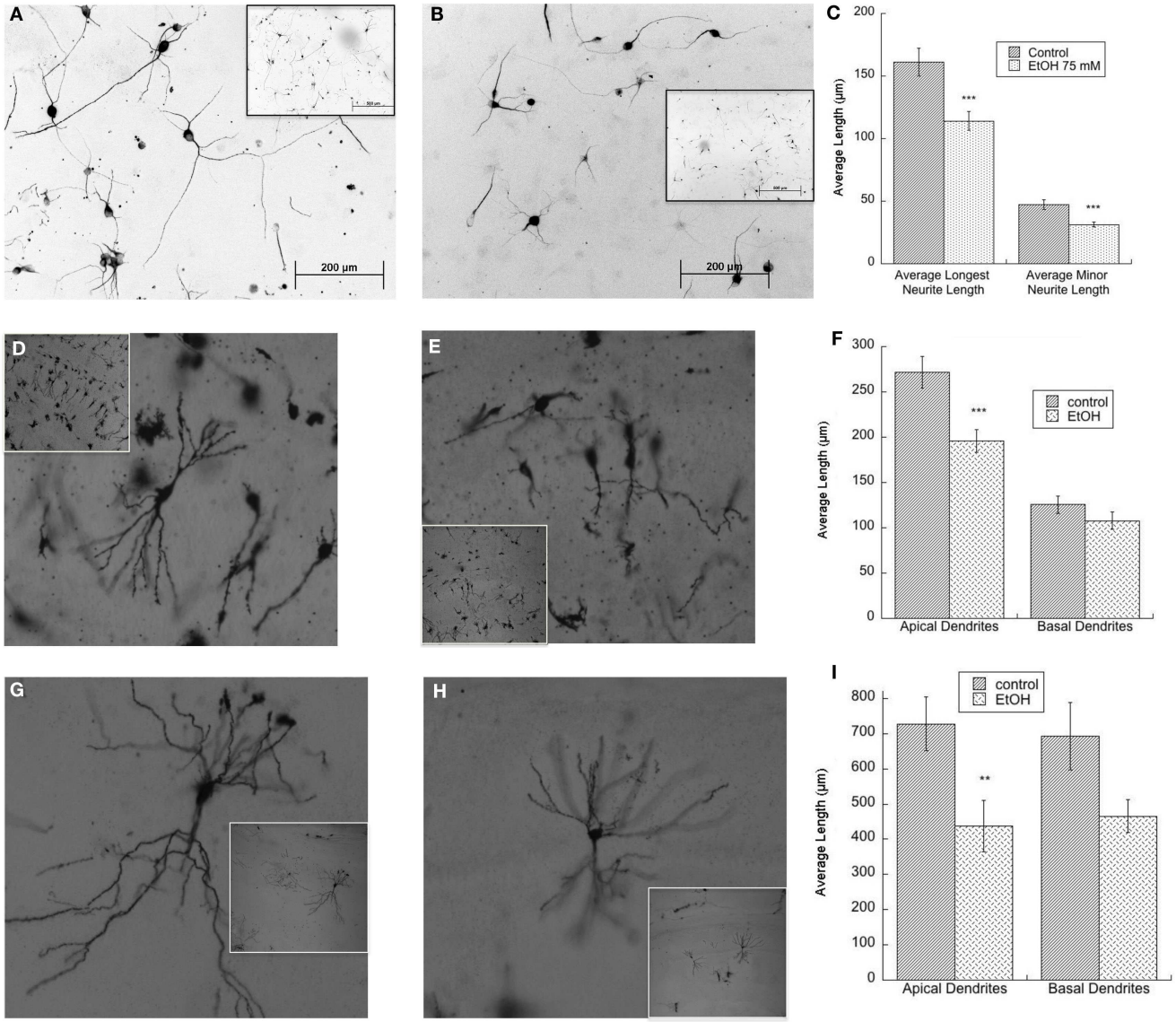


FIGURE 2 | (A–C) Ethanol-treated astrocytes inhibits hippocampal neuron neurite outgrowth. Hippocampal neurons plated on top of ethanol-pre-treated astrocytes (75 mM) display reduced neurite outgrowth. Shown are representative fields (20×) of neurons incubated with control **(A)** and ethanol-treated **(B)** astrocytes; *insets* show the same fields at a lower magnification (10×). **(C)** Quantification of the length of the longest neurite and of minor neurite in 60 cells per treatment was carried out using the software Image J. ***$p < 0.001$, Student's *t* test. **(D–F)** Neonatal ethanol exposure inhibits dendrite outgrowth in PD9 rats. Male rat pups were intubated with 5 g/kg ethanol or were sham (control) intubated from PD4 to PD9 and sacrificed on PD9. The brains were stained using the Golgi-Cox procedure. Representative CA1 neurons in control **(D)** and ethanol-exposed rats **(E)** are shown (10×); *insets* show the same fields at a lower magnification (4×). Dendrite length was measured using the software Neurolucida **(F)**. ***$p < 0.001$ by Student's *t* test. **(G–I)** Neonatal ethanol exposure reduces dendrite length in PD36 rats. Female rat pups were intubated with 5 g/kg/day of ethanol or were sham (control) intubated from PD4 to PD9 and sacrificed on PD36. Brains were stained using the Golgi-Cox procedure. Shown are representative CA1 hippocampal neurons in control **(G)** and ethanol-exposed rats **(H)** (10×); *insets* show the same fields at a lower magnification (4×). Dendrite length was measured using the software Neurolucida **(I)**. **$p < 0.01$, Student's *t* test.

which the two cell types are not in direct contact, an effect confirmed also in hippocampal slices (25, 35). In addition, in the absence of muscarinic receptor stimulation, ethanol-treated astrocytes displayed a reduced ability to foster neurite outgrowth when hippocampal pyramidal neurons and astrocytes are in direct contact (117) (**Figures 2A–C**). Reduced pyramidal neuron development is also observed in the hippocampus of postnatal day (PD) 9 and PD 36 rats exposed to ethanol between PD 4 and PD 9, a model of alcohol exposure mimicking the third trimester of human exposure (**Figures 2D–I**), in agreement with what reported by others in hippocampal and cortical pyramidal neurons (106, 109).

Ethanol inhibits astrocyte-mediated neurite outgrowth by profoundly affecting astrocyte secretion leading to the generation of an environment that is repressive of neuronal development. We have characterized several mechanisms by which ethanol affects astrocyte secretion:

(a) Ethanol reduces the release of neuritogenic ECM proteins laminin and fibronectin stimulated by muscarinic receptors in astrocytes (35), which may be due to the perturbation of the secretory pathway induced by ethanol in astrocytes (118).
(b) Ethanol induces a dysregulation of the plasminogen activator system, an important player in ECM proteolysis. Plasminogen is converted to the active proteolytic enzyme plasmin by two plasminogen activators: tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which are regulated by plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor superfamily. PAI-1 binds and inhibits plasminogen activators, thereby preventing the proteolytic activation of plasminogen to plasmin and the degradation of the ECM (119). We observed that ethanol inhibits the upregulation of PAI-1 induced by muscarinic receptor activation, therefore, increasing the degradation of EMC proteins laminin and fibronectin. The inhibitory effect of ethanol is in part mediated by ethanol-induced inhibition of the PLD signaling in astrocytes (**Figure 1**) (35).
(c) In addition, we found that ethanol increased the levels and release of tPA in astrocytes (120). Tissue-PA is upregulated by alcohol in the brain of animal models of both alcoholism and FASD, where it reduces the levels of laminin and causes neurodegeneration (121, 122). We found that the upregulation of tPA by ethanol is mediated by the epigenetic mechanism of DNA methylation. Indeed, ethanol inhibits DNMT activity and DNA methylation in the promoter region of tPA, thereby increasing tPA expression in astrocytes (120).
(d) Ethanol through the inhibition of arylsulfatase B (ARSB) activity increases the levels of chondroitin-4-sulfate and of the chondroitin sulfate proteoglycan neurocan, which are inhibitors of neurite outgrowth, in astrocytes *in vitro* and after neonatal alcohol exposure *in vivo* (117).

Together, the evidence discussed in this section underscores the role played by astrocytes in neuronal structural plasticity during brain development and outlines the novel and very important mechanism by which ethanol affects neuronal plasticity through alterations in astrocyte secretion.

ASTROCYTES, OXIDATIVE STRESS, AND FASD

Astrocytes are immunoresponsive cells. Several *in vitro* studies reported that ethanol induces oxidative stress in astrocytes in culture. Indeed, ethanol stimulates the formation of ROS, depletes glutathione, and upregulates cyclooxygenase 2 and inducible nitric oxide synthase expression via NF-κB activation in astrocytes (123, 124).

Ethanol activates Toll-like receptor 4/interleukin 1 receptor 1(TLR4/IL-1R1) signaling in astrocytes; inhibition of TLR4 and IL-1R1 abolishes ethanol-induced NF-κB and AP-1 activation, inducible nitric oxide synthase, and cyclooxygenase-2 upregulation indicating that these receptors mediate ethanol-induced inflammatory events in astrocytes (125). On the other hand, cortical neurons are more sensitive than astrocytes to ethanol and undergo apoptotic cell death mediated by increased ROS production and GSH depletion; attenuated neuronal cell death and reduced GSH depletion was observed when neurons are co-cultured with astrocytes (126, 127).

ASTROCYTES AND BRAIN LIPID HOMEOSTASIS IN FASD

The maintenance of optimal cholesterol levels is essential to brain development. As in the periphery, cholesterol is circulated in the brain associated with lipoproteins, which are produced by astrocytes; astrocytes and microglia, but not neurons, also express apolipoprotein E (apo E). Lipoproteins produced and released by astrocytes are discoidal in shape and contain apo E, phospholipids, and cholesterol, but lack in the core lipids (cholesterol esters or triglycerides). In contrast, lipoproteins found in the cerebrospinal fluid are round, contain a cholesterol ester core, and are similar to plasma high-density lipoproteins (HDL) (128, 129). An important mechanism of brain cholesterol clearance involves cholesterol efflux from brain cells to astrocyte-released lipoproteins, which exit the brain after passing from the brain parenchyma into the cerebrospinal fluid and across the blood–brain barrier (130–132).

We have been investigating the hypothesis that ethanol increases lipoprotein release from astrocytes leading to increased cholesterol clearance and reduced levels of cholesterol in the whole brain (133). The transporter ABCA1 (ATP-binding cassette-A1) is essential for the generation of nascent lipoproteins in astrocytes, subsequent cholesterol efflux is mediated by ABCG1 and ABCG4 transporters and lead to the lipidation and remodeling of nascent, lipid-poor lipoproteins (134–136).

We have shown that ethanol increases ABCA1 and ABCG1 levels, induces cholesterol efflux, and reduces cholesterol levels in primary rat astrocytes in culture (137). Interestingly, isotretinoin, which causes developmental effects similar to ethanol, induces ABCA1 and ABCG1 expression, increases cholesterol efflux, and decreases cholesterol content in astrocytes similarly to ethanol (137, 138), suggesting a common mechanism of teratogenesis (133). These observations were also confirmed in *in vivo* FASD models. Indeed, neonatal alcohol exposure increases cortical levels of ABCA1 (137); furthermore, prenatal alcohol exposure upregulates ABCA1 and ABCG1 and reduces the levels of cholesterol in the neocortex of GD 21 female fetuses (139). **Figure 3** shows a proposed model of the interactions of astrocytes and neurons in cholesterol homeostasis and the effects of ethanol.

ASTROCYTE DIFFERENTIATION IN FASD

Published literature support the notion that the survival of progenitor cells and their differentiation into astrocytes is inhibited by ethanol leading to an overall decreased astrocyte population, which may strongly affect neuronal development and survival given the trophic role played by astrocytes.

Ethanol reduces the survival of neural progenitors from human embryonic stem cells and their differentiation into astrocytes (140, 141). Furthermore, ethanol exposure during embryogenesis reduces the telencephalic radial glia progenitor pool and its

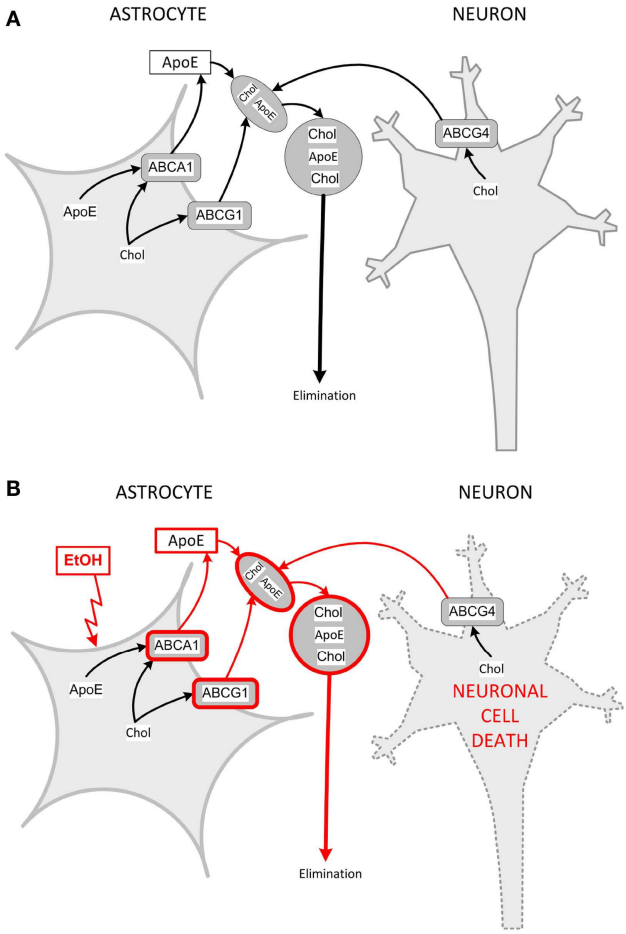


FIGURE 3 | Model for interactions of astrocytes and neurons in cholesterol clearance and dysregulation by ethanol. **(A)** Cholesterol is produced by both astrocytes and neurons. Astrocytes produce and release nascent, lipid-poor lipoproteins through the lipidation of apoE via the membrane-bound ABCA1. In the brain parenchyma, nascent lipoproteins stimulate cholesterol efflux through their interaction with ABCA1 and ABCG1 in astrocytes and ABCG4 in neurons. Lipoprotein-associated cholesterol exits the brain through the cerebrospinal fluid. **(B)** Ethanol upregulates ABCA1 and ABCG1 in astrocytes, and increases the formation of astrocyte lipoproteins, which extract more cholesterol from astrocytes and neurons (pathways upregulated by ethanol are in red). Neurons are sensitive to changes in cholesterol content and, upon protracted induction of cholesterol efflux, decrease their cholesterol content and undergo cell death. Chol, cholesterol.

differentiation into neurons and astrocytes (142). Ethanol inhibits precursor cell proliferation and astrogliogenesis also in a rodent neurosphere culture model (143).

OLIGODENDROCYTES AND FASD

Most of the earlier studies on the effects of fetal alcohol on myelination and oligodendrocytes have been carried out between the late 1970s and the early 1990s followed by a paucity of papers emerging for more than a decade. However, the last few years were characterized by a resurrected interest in the effects of alcohol on myelination during brain development. This was triggered by advancements in imaging techniques allowing for better evaluation of white matter damage in FASD individuals.

Oligodendrocyte differentiation and myelination in humans occur mostly after birth; however, myelination is affected by *in utero* alcohol exposure indicating that fetal alcohol effects on OPCs results in altered oligodendrocyte development and myelination. Indeed, imaging studies have found global white matter reduction and white matter abnormalities in children and adolescents with FASDs (144, 145). Myelination is affected in several *in vivo* FASD models. In developing rat brains, exposure to ethanol alters myelin ultrastructure and delays myelination (146–148). It was more recently reported that myelin is disrupted and oligodendrocyte morphology is altered also in a third-trimester equivalent sheep model of FASD (149).

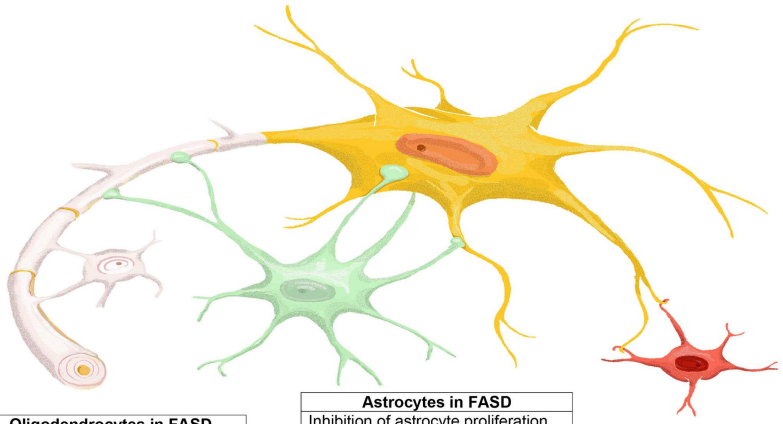
While abnormalities and delays in the biochemical profile of myelin (150–152) and in myelin protein synthesis were reported following prenatal alcohol exposure in rats, more dramatic effects were obtained after ethanol exposure during the third-trimester equivalent (i.e., the first 10 PDs), when oligodendrocytes begin maturation and myelination (148). Several studies report delayed or reduced expression of oligodendrocyte proteins suggesting delayed differentiation. Ethanol delays the expression of myelin basic protein (MBP) and the maturation of oligodendrocytes cultured from PD 1–2 rats prenatally exposed to ethanol (153). Postnatal ethanol exposure reduces the levels of MBP and myelin-associated glycoprotein also in the cerebellum of PD15 rats (154). Direct exposure of oligodendrocytes in culture to ethanol confirms an inhibited expression of MPB (155). Recently, widespread oligodendrocyte apoptosis has been reported in the white matter regions of the fetal brain of monkeys exposed to high alcohol levels during the equivalent of the third trimester of human gestation (156).

Hypoplasia of the optic nerve is common in individuals with FAS and may be responsible for their reduced visual function (157). Several alterations in the optic nerve myelin have been reported in animal models of FASD, including permanent reduction in myelin thickness, fewer myelinated axons, aberrant myelin sheaths, and myelin acquisition, which may account for fetal alcohol-induced hypoplasia of the optic nerve (147, 158–160).

Together, these studies clearly indicate that oligodendrocytes development and survival are affected by fetal alcohol leading to altered myelination, which may have a great impact on axonal size and ability to effectively transmit action potentials. Further research is required to fully understand the mechanisms involved in the effects of prenatal ethanol on oligodendrocyte development and myelination.

MICROGLIA AND FASD

Microglial cells can be directly activated by alcohol. Indeed, ethanol, by activating TLR2 and TLR4 signaling in microglia, triggers phagocytosis and production of ROS and cytokines, factors that contributes to inflammation and cortical neuron apoptosis (161, 162). Microglia play also a role in alcohol-induced apoptosis of developing hypothalamic neurons. Indeed, ethanol increases the release of inflammatory cytokines form microglia and induces oxidative stress and decreases the intracellular levels of cAMP and BDNF in hypothalamic neurons co-cultured



Oligodendrocytes in FASD
White matter reduction and abnormalities
Delayed myelination, reduced myelin thickness, ultrastructural myelin abnormalities
Abnormalities in biochemical profile of myelin
Delayed/reduced oligodendrocytes differentiation and expression of MBP and myelin associated glycoprotein
Oligodendrocyte apoptosis

Astrocytes in FASD
Inhibition of astrocyte proliferation
Reduced release of factors promoting neurite outgrowth from astrocytes
Increased release of factors inhibiting neurite outgrowth from astrocytes
Astrocyte activation of TLR4-IL1R1, NOS and cyclooxygenase 2 induction, GSH depletion
Cholesterol homeostasis alterations in astrocytes
Reduced progenitor cell survival and astrocyte differentiation

Microglia in FASD
Increased microglia activation
Increased microglia-mediated hypothalamic neuron death
Increased microglia death

FIGURE 4 | Representation of the main effects exerted by ethanol on astrocytes, oligodendrocytes, and microglia that may play a role in the neuropathology of FASD.

with ethanol-treated microglia (163–165); Ethanol also induces microglia cell death both *in vitro* and after neonatal alcohol *exposure in vivo*, effects that are prevented by a PPRγ agonist (166). The effects of alcohol on neuroinflammation processes involving microglia activation in fetal, adolescent, and adult brain are the topic of several recent review articles and book chapters (167–170).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Recent studies indicate the essential role glial cells play in the developing brain. For example, growth factors released by astrocytes guide neuritogenesis and support neuronal survival, microglia mediate synaptic pruning, and oligodendrocytes regulate axonal size, function, and survival. In this way, the development of brain circuits depends on glia–glia and glia–neuron interactions. In addition, several mechanisms underlying many neurodegenerative and neurodevelopmental diseases are non-cell-autonomous and involve glia–neuron interactions (171).

In this review, we summarize published evidence that glia–neuron interactions play an essential role also in the pathophysiology of FASD. Astrocytes, oligodendrocytes, and microglia are all highly affected by *in utero* alcohol exposure as summarized in **Figure 4**. The impact of alcohol exposure on the brain should not be considered as the sum of the isolated action of ethanol on neurons, astrocytes, oligodendrocytes, and microglia. Indeed, it is clear that alcohol not only affects signaling pathways within a cell type but also strongly alters the ability of glial types to send information to neurons and *vice versa*, therefore, greatly disrupting the synchronized series of events that lead to the correct development of the brain and the proper formation of the brain circuit architecture. Thus, it is imperative that novel therapeutic strategies for the treatment or prevention of the neurodevelopmental effects of ethanol target not only the functions of individual cell types but also mechanisms of glia–glia and glia–neuron communications.

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