



Published in final edited form as:

Trends Neurosci. 2012 May ; 35(5): 284–292. doi:10.1016/j.tins.2012.01.006.

Does moderate drinking harm the fetal brain? Insights from animal models

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Abstract

Although public health campaigns advise pregnant women to abstain from ethanol, drinking during pregnancy is pervasive. Here, we highlight recent studies that have clearly demonstrated long-lasting neurobehavioral deficits in offspring of laboratory animals exposed to moderate levels of ethanol during development. Alterations in learning, memory, motor coordination, social behavior, and stress responses were identified in these animals. Increased vulnerability to substance abuse was also demonstrated. These behavioral alterations have been associated with impairments in neurotransmitter systems, neuromodulators, and/or synaptic plasticity in several brain regions. With this review, we hope to contribute to a better appreciation of the potential effects of developmental exposure to moderate ethanol levels, leading to better interventions aimed at relieving fetal alcohol spectrum disorders.

Keywords

alcohol; ethanol; neurotransmission; fetal alcohol syndrome; animal model; pregnancy

Introduction

Exposure to ethanol during pregnancy produces fetal alcohol spectrum disorders (FASDs), a major public health problem with an estimated prevalence of 1-5% in the United States and Western Europe, and an even higher prevalence in other regions, including South Africa [1]. The high prevalence of FASDs is not surprising given that a significant percentage of pregnant women consume ethanol (e.g., 10-20% in the U.S.A., 40% in Uruguay; 50% in some parts of Italy) [2-4]. The most severe form of FASDs is fetal alcohol syndrome (FAS), characterized by growth retardation, facial abnormalities, and neurobehavioral alterations. FAS is often associated with heavy ethanol consumption throughout pregnancy, including binge drinking. Most patients with FASDs exhibit only a subset of the characteristics of FAS, such as cognitive and behavioral deficits, in the absence of facial alterations.

Although it is widely accepted that exposure to high doses of ethanol has long-lasting detrimental effects on brain development, the case for moderate exposure (Box 1) remains controversial [5-7]. On one side are a number of studies suggesting that moderate prenatal

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alcohol exposure (MPAE) is associated with a higher incidence of behavioral and cognitive problems during childhood and/or adolescence, including mood disorders, deficits in working memory and attention, increases in aggression, and alterations in peer relationships [8-12]. Conversely, other studies have shown that MPAE is not associated with an increased incidence of social, motor, or emotional problems [13-17]. Reasons for the discrepancies between these studies are likely to include differences in methodology, sample demographic characteristics, confounding variables (e.g., stress, genetic factors, socioeconomic status, nutrition, or co-exposure to other harmful substances), and accurate determination of drinking patterns [7] (Box 1).

Although human epidemiological studies are inconclusive regarding the impact of MPAE, primate and rodent models of FASDs have convincingly demonstrated significant effects in several brain regions. In this review, we focus on studies completed in the last decade where animals were exposed *in vivo* to blood alcohol levels (BALs) ≤ 0.17 g/dl, which have been assumed to model human MPAE (Box 1; for reference, the U.S. legal intoxication limit is 0.08 g/dl = 17.4 mM). We elected to concentrate on studies with BALs up to approximately twice the legal intoxication limit in the U.S. based on a recent computational modeling study indicating that significantly higher BALs are required to produce neurodevelopmental effects in rodents than in primates [18]. Studies discussed here used a variety of exposure paradigms (Fig 1) to assess ethanol's effects across different brain regions. The reader is referred to more comprehensive reviews for additional information on FASD, including studies that have used other modes of exposure and higher ethanol doses [19-24].

Hippocampal Formation

Glutamatergic and Histaminergic Transmission

Studies with rodents have provided strong evidence indicating that MPAE impairs hippocampal-dependent memory. MPAE adult rat offspring (liquid diet during pregnancy; BALs of 0.03 and 0.08 g/dl) displayed impaired performance on a moving platform version of the Morris Water Task [25]. Furthermore, hippocampal slice experiments from prenatally exposed rats revealed a reduction in activity-dependent potentiation of evoked [3 H]-D-aspartate release, suggesting that abnormalities in glutamatergic plasticity may underlie the behavioral alterations [25].

A more recent study confirmed that voluntary drinking in pregnant rats (BAL = 0.08 g/dl) induced long-lasting learning and memory deficits in their offspring [26]. MPAE adult rat offspring exhibited alterations in hippocampal-dependent memory assessed using both the Morris Water Task and 1-trial contextual fear conditioning [26] (Glossary) (Fig 2A-B). *In vivo* electrophysiological experiments under urethane anesthesia demonstrated a MPAE-induced deficit in glutamatergic long-term potentiation (LTP) in the dentate gyrus (DG), providing a possible underlying mechanism for observed alterations in spatial memory [27] (Fig 2C). As discussed below, histamine neurotransmission may also be involved.

Histamine is a monoamine neurotransmitter synthesized by the hypothalamic tuberomammillary nuclei neurons that project to several brain regions, including the DG. Histamine has been demonstrated to regulate the sleep-wake cycle, feeding behavior, and memory formation [28]. The neuronal actions of histamine are mediated by four subtypes of receptors (H1-4). H3 receptors have generated significant interest, as these could be potential drug targets for the treatment of several neuropsychiatric disorders [28]. The investigational H3 antagonist, ABT-239, ameliorated the MPAE-induced deficits in the Morris Water Task, contextual fear conditioning, and DG LTP (Fig 2), suggesting that this agent may be potentially useful in the treatment of FASDs [26, 27]. Although the mechanism of ABT-239 action is complex, it possibly involves facilitation of glutamate,

acetylcholine and/or histamine release via inhibition of presynaptic H3 receptors. These findings are especially exciting as there are limited existing pharmacological treatments against FASDs.

Studies have also observed deficits in hippocampal-dependent behavioral tests (contextual fear conditioning, trace fear conditioning, and radial-arm maze tasks) in MPAE adult mouse offspring (voluntary drinking paradigm; BAL = 0.09-0.12 g/dl) [29, 30]. Immunohistochemical experiments showed that MPAE (BAL = 0.08 g/dl) decreases NMDA receptor (NMDAR)-dependent activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the DG, but not the CA1 and CA3 hippocampal regions, indicating that alterations in the DG may underlie the behavioral deficits [31]. Importantly, ERK1/2 activation is involved in some forms of NMDAR-dependent LTP in the DG [32].

Moderate ethanol exposure of rats during the 3rd trimester-equivalent [postnatal days 1-8 (P1-8); vapor chambers; BAL = 0.17 g/dl] was also shown to persistently alter hippocampal-dependent learning [33]. These behavioral deficits were associated with increases in levels of vesicular glutamate transporter 1, excitatory amino acid transporters 1 and 3, and GluN2A-D NMDAR subunits in the hippocampal formation and/or neocortex [33].

GABAergic Plasticity

During the 3rd trimester-equivalent, GABAergic synapses are actively refined by synaptic plasticity-dependent mechanisms. In the hippocampus, activity-dependent retrograde release of brain-derived neurotrophic factor (BDNF) from CA3 pyramidal neuron dendrites causes persistent potentiation of GABAergic interneuron-CA3 pyramidal neuron synapses, a phenomenon known as GABA_A-LTP [34]. It was found that moderate exposure of rats during the 3rd trimester-equivalent (vapor chambers; P2-6; lowest BAL tested = 0.02 g/dl) inhibited GABA_A-LTP [35]. The mechanism of action of ethanol is hypothesized to involve persistent inhibition of L-type voltage-gated Ca²⁺ channels, leading to a reduction in dendritic release of BDNF [35]. These findings indicate that ethanol exposure can potentially affect GABAergic transmission and plasticity during development, which could partially be responsible for the long-lasting alterations in neuronal circuits associated with FASDs. It should be investigated whether ethanol exposure during the 3rd trimester-equivalent persistently decreases GABAergic transmission altering the balance of excitatory and inhibitory inputs.

Adult Neurogenesis

A number of studies suggest that alterations in adult neurogenesis could contribute to the pathophysiology of FASDs [23]. The effects of MPAE on adult neurogenesis were first characterized in mice (voluntary drinking paradigm; BAL = 0.12 g/dl) [36]. This study found no significant difference in the survival or differentiation of progenitor cells in the DG of adult offspring under standard housing conditions, but did report that MPAE blocked an environmental enrichment-induced increase in survival of progenitor cells. Conversely, a recent study found reduced generation of new neurons and glial cells in MPAE rat female adult offspring (liquid diet paradigm; BAL = 0.14 g/dl) [37]. These findings indicate that the effects of MPAE on adult neurogenesis in the DG depend on the method of ethanol exposure and the characteristics of the animal model (i.e., age, species, and gender).

MPAE also has profound consequences on neurogenesis in other areas of the brain. MPAE adult mice (voluntary drinking; BAL = 0.12 g/dl) were shown to be significantly less capable of discriminating between two odors versus controls [38]. Further experiments revealed that ethanol-exposed mice have a reduced number of olfactory bulb precursor

neurons and fewer new neurons in the olfactory bulb granule cell layer during the early postnatal period.

The mechanisms responsible for the long-lasting effects of MPAE on neurogenesis are presently unknown but could involve intrinsic alterations in the progenitor cells themselves and/or deficits in the stem cell niche. A recent study found evidence of reduced intrinsic neurogenic capacity of neural progenitor cells derived from the adult subventricular zone of MPAE mice [39]. Alternatively, MPAE-induced alterations in neurogenesis may be secondary to chronic stress [40]. It has been shown that MPAE programs the hypothalamic-pituitary-adrenal (HPA) axis, increasing its activity, leading to sensitization to stressors throughout life. This effect increases vulnerability to stress-related disorders, such as anxiety and/or depression, which could be mediated by alterations in adult neurogenesis [41]. MPAE (liquid diet; BAL 0.14 g/dl) alone was found to inhibit adult neurogenesis in the DG, and restraint stress could not further reduce neurogenesis unlike in control rats [42]. One possibility is that the MPAE-induced increase of HPA axis activity reduces neurogenesis to a degree that cannot be dampened further by additional stress. Clearly, future studies are needed to understand the role of HPA axis alterations in the neurogenesis deficits associated with MPAE.

Thyroid Hormone and Retinoic Acid

The thyroid hormone, triiodothyronine (T3), is degraded by iodothyronine deiodinase-III, which is encoded by an imprinted gene, leading to differential expression of maternal vs. paternal genes. A recent study demonstrated that MPAE (liquid diet; BAL = 0.12) reduced the paternal and total expression of iodothyronine deiodinase-III in the hippocampus of fetal and adult rat males only, leading to an increase in T3 levels [43]. These alterations were linked to deficits in the Morris Water Task in adult male rats. In the frontal cortex, MPAE increased total expression of iodothyronine deiodinase-III and reduced T3 levels in both genders. The effects of MPAE in the hippocampus and frontal cortex were dependent on the genetic background, as offspring of Sprague-Dawley mothers crossed with Brown-Norway fathers exhibited MPAE-induced alterations but not offspring of reciprocal crosses. This study indicates that gender-, brain region-, and genotype-specific imprinting processes could interact with MPAE to produce long-lasting deficits in hippocampal-dependent spatial memory.

MPAE (liquid diet; BAL = 0.01-0.12 g/dl) has also been shown to dramatically increase all-*trans* retinoic acid (RA) levels both in the hippocampus and neocortex of embryonic day 19 (E19) fetuses [44]. Given that RA is an important regulator of adult neurogenesis and neurotransmission [45], it should be investigated whether alterations in the levels of this biologically active form of vitamin A can be an additional factor underlying the neurobehavioral alterations induced by MPAE.

Cerebellum

The cerebellum—involved in motor coordination and learning, as well as cognitive and emotional functions—has been shown to be affected by MPAE. Exposure of pregnant macaque monkeys to moderate levels of ethanol (voluntary drinking; BAL = 0.02-0.05 g/dl) caused alterations in motor coordination and response speed in infant offspring (reviewed in [21]). Similarly, MPAE mouse offspring (forced drinking; BAL = 0.07 g/dl) exhibited significant motor impairments determined by rotarod and runway assays (at P 24) in addition to deficits in motor learning as measured by eyeblink conditioning [46]. *In vivo* electrophysiological recordings showed that spontaneous firing of Purkinje neurons is significantly increased and accompanied by fast local field potential oscillations, which may be associated with disruptions in information flow within the cerebellar cortex. *In vitro*

cerebellar slice recordings showed reduced glutamate release at parallel fiber (i.e., axons from cerebellar granule cells) to Purkinje cell synapses [46]. Long-term depression (LTD) was shifted toward LTP at these synapses, an effect associated with a reduction in both protein kinase C- γ expression and voltage-gated Ca^{2+} channel function [46].

MPAE has also been shown to produce abnormalities in other cerebellar neuronal populations. Exposure of rats through gestation and weaning (voluntary drinking; BAL = 0.07 g/dl) increased soma size and dendritic length in cerebellar granule cells, and delayed maturation of Bergmann glia [47]. Moreover, a recent study reported alterations in cerebellar granule cell migration in a mouse model of ethanol exposure during the 3rd trimester-equivalent (1 g/kg intraperitoneal injection at P10; BAL = 0.07 g/dl) [48]. In addition, 3rd trimester-equivalent exposure (1-2 g/kg/day P2-11; intragastric intubation; BAL = 0.05-0.15 g/dl) impaired eyeblink conditioning in adult rats, which could be related to alterations in deep cerebellar nuclear neurons [49]. Collectively, these studies clearly indicate that the developing cerebellum is a sensitive target of MPAE.

Neocortex

Several brain regions have been linked to social behavior in rats, including the frontal cortex. Long-lasting alterations in social behavior were recently demonstrated in MPAE adult rats (voluntary drinking; BAL = 0.08 g/dl) [50]. Impairments in social behavior were more consistently observed in adult male rats in response to a new cage mate. Ethanol exposed rats exhibited increases in anogenital and body sniffing (social investigation), and wrestling behavior (either aggressive or playful behavior). Exposure to a new cage mate increased expression of activity-related immediate early genes [c-fos and/or activity-regulated cytoskeletal (Arc) gene] in the agranular insular cortex (equivalent to the orbital prefrontal cortex in primates), the prelimbic cortex, and lateral orbital area in control offspring but this effect was not observed in MPAE offspring [50, 51]. Furthermore, when control rats were exposed to various cage-mates, they exhibited an increase in dendritic length and spine density in the agranular insular cortex. Conversely, MPAE offspring exhibited an increase in dendritic length with no change in spine density. It should be noted that another study with rats demonstrated that MPAE (forced drinking; BAL = 0.015-0.04 g/dl) results in deficits in social recognition memory, which were correlated with neocortical and hippocampal increases in levels of an endogenous neurosteroid (3 α -hydroxy, 5 α -pregnan-20-one) that potently modulates GABA_A receptor (GABA_AR) function [52]. These studies suggest that MPAE causes long-lasting alterations in social behaviors that could possibly be a consequence of synaptic plasticity deficits in the cerebral cortex.

MPAE has also been shown to affect neocortical GABAergic neuron development. During early development in mice (E0.5-14.5), it was found that MPAE (liquid diet; BAL = 0.03 g/dL) increases the density of GABAergic interneurons in the neocortex [53]. Ethanol exposure caused premature migration of medial ganglionic eminence-derived GABAergic neurons into the neocortex and also promoted differentiation of these neurons. Studies with slice co-cultures showed that ethanol increases ambient GABA levels, an effect that was also demonstrated in acute slices by monitoring tonic GABAergic currents in migrating cells [53]. In addition, it was demonstrated that MPAE increases sensitivity of GABA_ARs to exogenous GABA, suggesting a change in the subunit composition of these receptors. Related to this study, MPAE (liquid diet during pre-conception, pregnancy and gestation; BAL=0.09 g/dl) was recently shown to decrease cortical thickness in rat fetuses and/or neonates, an effect that could be a consequence of alterations in radial glia [resulting from reduced expression of the paired box 6 (Pax6) transcription factor], delayed neuronal migration, and decreased numbers of neuroblasts [54].

Exposure to ethanol during the 3rd trimester equivalent was recently demonstrated to produce long-lasting impairments in the excitability of layer 5 pyramidal neurons of the rat neocortex [55]. Rats were exposed to ethanol during P2-6 via vapor chambers (lowest range of BAL = 0.15 g/dl) and slice electrophysiological recordings were performed at P30-60. Depolarizing current injection resulted in dendritic spikes with significantly lower frequency and duration in neurons from ethanol exposed animals. The somatic response to dendritic current injection was also reduced in neurons from the ethanol group. Imaging studies revealed that dendritic Ca²⁺ transients mediated by voltage-gated Ca²⁺ channels were reduced in neurons from ethanol treated animals. Taken together with the results of other studies (see hippocampal and cerebellar sections) [35, 46], these findings further support the possibility that voltage-gated Ca²⁺ channels are important targets of ethanol exposure during development.

Dopaminergic and Serotonergic Systems

Dopaminergic System

The dopaminergic system has been tightly linked to drug abuse and a number of studies have demonstrated that MPAE increases vulnerability to ethanol and other abused substances. Behavioral studies have shown that MPAE increases: 1) orienting responses towards ethanol odor, 2) palatability and consumption of ethanol, and 3) reinforcing effects of ethanol [56, 57]. Consonant with these results, ethanol exposure during pregnancy and lactation (forced drinking; maternal BAL= 0.009-0.12 g/dl; suckling offspring BAL = 0.019 g/dl), increased preference and consumption of ethanol-containing solutions in adult rat offspring [58, 59]. Adult rats from the ethanol group also exhibited increased cocaine and amphetamine consumption after cocaine exposure, as well as enhanced conditioned place preference for ethanol and cocaine. Interestingly, these animals had an increased sensitivity to the anxiolytic effects of acute ethanol exposure and stimulant effects of ethanol, cocaine, and amphetamine (but decreased sensitivity to the sedative effects of ethanol). These animals exhibited complex age-dependent changes in mRNA expression for BDNF, the GluN1 NMDAR subunit, type-1 cannabinoid receptor, and dopamine receptors (D1 and D2) in the neocortex, thalamus, striatum, hippocampus and/or cerebellum [58, 59]. Furthermore, reductions in radioligand binding to D1 and D2 receptors, and/or dopamine transporters were also evident in the striatum of ethanol-exposed rats (at 1-2 months of age) [58].

Other studies have examined the effect of MPAE on ethanol consumption and preference. It was recently reported that MPAE guinea pigs (continuous-access voluntary drinking; expected BAL = 0.05 g/dl) showed increased ethanol preference [60]. However, another study reached a different conclusion: prenatal co-exposure of rats to ethanol (liquid diet; BAL = 0.1 g/dl) and nicotine (osmotic minipump; 3-6 mg/kg/day) decreased ethanol preference in adolescent males, and reduced both consumption and preference in adult female rats [61]. This raises the possibility that prenatal co-exposure to ethanol and other drugs of abuse has a different effect on drug-seeking behavior compared to ethanol exposure alone.

Studies with primates have also revealed MPAE-induced dopaminergic alterations. Pregnant rhesus monkeys were exposed to ethanol (voluntary drinking; BAL = 0.02-0.05 g/dl) during early pregnancy (gestational days 0-50), middle-late pregnancy (gestational days 50-135) or throughout pregnancy (gestational days 0-135) [62]. Positron emission tomography studies revealed that exposure during early pregnancy and throughout pregnancy reduces striatal dopaminergic function in young adult offspring (as measured by the ratio of striatal D2 receptor binding over dopamine synthesis). Interestingly, mid-late gestation exposure had the opposite effect indicating that the timing of prenatal exposure dictates the outcome. A subsequent study by the same group of investigators showed a reduction in aversive

responses to repetitive tactile stimulation in young adult monkeys exposed to ethanol throughout gestation that could be related to changes in dopaminergic function in the striatum [63]. Given the regulatory role of the striatum on the function of other brain regions, this alteration in striatal dopaminergic signaling could result in impaired control of sensory input [21].

The above-described studies with primates indicate that MPAE during late pregnancy could significantly affect striatal function. In general agreement with these results, it was reported that exposure to moderate ethanol levels (0.63 g/kg subcutaneously at P 7; BAL=0.05 g/dl) increases caspase-3 activation in the mouse caudate nucleus (but not the thalamus) 4 hr after injection, suggesting activation of apoptotic pathways in this brain region [64].

Serotonergic System

Human and animal studies have implicated serotonin neurotransmitter system alterations in the pathophysiology of FASDs [65, 66]. MPAE (Liquid diet; BAL=0.017-0.14 g/dl) was shown to impair neural tube midline development in fetal mice, which negatively impacts the development of serotonergic neurons in the raphe nucleus of the brainstem [67]. A recent study with primates demonstrated a gene-environment interaction between the serotonin transporter gene-linked polymorphic region (5-HTTLPR) and MPAE. A variation in this region has been identified in humans resulting in long and short forms of the serotonin transporter (SERT), the former being associated with higher expression of SERT [68]. Long and short forms of this region have also been identified in rhesus monkeys and the effect of MPAE (voluntary drinking; BAL = 0.02-0.05 g/dl) has been studied [69]. In neonatal MPAE offspring, monkeys with the short form were more irritable, and had significant adrenocorticotrophic hormone and cortisol increases in response to mother-infant separation. These findings suggest that having the short variant of 5-HTTLPR may result in altered responsiveness of the serotonin system to modulation by stress hormones, an effect that is exacerbated by MPAE. Alternatively, altered serotonergic modulation of the HPA axis could be the cause of the increased responsiveness to stress in these animals, a conclusion that is generally supported by studies with rodents [70]. Importantly, this study further highlights the role that genetics can play in modulating susceptibility to the effects of MPAE [43].

Another effect of MPAE on the serotonin system was identified in respiratory centers in the brain stem [71]. In brain stem slices from control rats, the amplitude of hypoglossal nerve rootlet burst activity can be persistently increased in response to brief episodes of anoxia, a phenomenon that is known as respiratory LTP. In slices from rats exposed to ethanol during pregnancy and lactation (forced drinking; maternal BAL = 0.08 g/dl; nursing pup BAL = 0.019 g/dl), respiratory LTP was converted into LTD. Serotonin accumulates during hypoxia and plays a critical role in the induction of respiratory long-term facilitation. Therefore, the authors measured expression of serotonin (5-HT) $2_A/2_C$ receptors, which have been implicated in this process. It was shown that mRNA levels for these receptors were decreased in the ethanol group and higher concentrations of serotonin were required to induce facilitation of respiration [70]. These findings may be therapeutically significant, as alterations in brain stem serotonergic signaling could explain the higher incidence of sudden infant death syndrome in patients with FASDs [72].

Concluding Remarks

The collective evidence from the animal studies reviewed above strongly suggests that MPAE can persistently alter multiple neurotransmitter and neuromodulatory systems throughout the brain, leading to significant neurobehavioral alterations in offspring (Fig 3). Some studies showed significant effects at BALs as low as 0.02 g/dl, which could be

achieved after consumption of just 1 drink/hr (Box 1). Several mechanisms that could account for the behavioral effects of ethanol have been demonstrated, including alterations in neuronal migration, adult neurogenesis, neurotransmitter receptor function, synaptic plasticity, and intracellular signaling pathways. Importantly, modulation of histamine neurotransmission has been identified as having potentially beneficial effects to reverse some of the deficits induced by MPAE.

The findings of the above-reviewed preclinical studies could be translated into the clinic in several ways. First, public educational campaigns on the potential effects of MPAE should be widely implemented and training on this important issue should be provided to medical students, residents, and other health care professionals involved in the care of pregnant women, as these professionals may be unaware of the potential dangers of MPAE [73]. In addition, public health campaigns against FASDs should include information on studies of the effects of MPAE. Second, health care professionals should be made aware of the current guidelines/methods for ethanol consumption screening during antenatal and prenatal care visits, and interventions that could aid in the prevention of drinking during pregnancy [74]. Third, development of laboratory tests that can objectively assess MPAE should continue to be actively pursued [75]. Fourth, offspring of mothers who consumed ethanol at moderate levels should be assessed for cognitive alterations at multiple levels and at different stages of development, as deficits could be significantly ameliorated by early behavioral or pharmacological interventions [76]. Although further research is required to fully understand the potential consequences of MPAE (Box 2), studies with animal models clearly indicate that MPAE can significantly affect brain development. Clinicians should take the findings of these studies into account when advising women about ethanol consumption during pregnancy and should also consider MPAE as a potential cause of neurobehavioral disorders identified during adulthood.

Acknowledgments

Supported by National Institutes of Health grants RO1-AA015614, RO1-AA014973, T32-AA014127, K12-GM088021 and P20-AA17068. We are grateful to Dan Savage, Kevin Caldwell, and Rafael Varaschin for critically reading the manuscript.

Glossary

Brain reward system	a neuronal circuit that includes dopaminergic neurons in the ventral tegmental area projecting to several target regions including the nucleus accumbens, prefrontal cortex, and amygdala.
Contextual fear conditioning	a behavioral test in which animals learn to associate fear with spatial environmental cues.
Eyeblink conditioning	a behavioral test in which animals learn to associate auditory or visual stimuli with a stimulus that induces an eyeblink response (e.g., airpuff to the cornea).
Positron emission tomography	an imaging technique that is capable of detecting radioactive chemicals in brain and other organs.
Stem cell niche	microenvironment that regulates self-renewal, differentiation, and maturation of stem cells.

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Box 1. What is moderate drinking?

The definition of both the content of ethanol in a standard drink and moderate drinking varies by country (<http://www.icap.org/table/Internationaldrinkingguidelines>). In general, government guidelines recommend that women consume no more than 10-40 g of ethanol/day. In the U.S.A., 1 standard drink (14 g of ethanol)/day is considered moderate consumption for non-pregnant women [77]. BALs depend on many factors, including gender, weight, and presence of food in the stomach. Consumption of 28 g of ethanol in 1 hr by a 60 kg non-pregnant woman is estimated to yield a BAL \approx 0.06 g/dl (<http://www.dot.wisconsin.gov/safety/motorist/drunksdriving/calculator.htm>). However, the effect of pregnancy on ethanol metabolism is not fully understood, with studies showing increased metabolism in rats [78] or decreased metabolism and accumulation in the amniotic fluid in guinea pigs [79, 80]. A study with women revealed faster elimination kinetics of ethanol from maternal blood during the early 2nd trimester in comparison to non-pregnant women; however, this study also found a slower rate of ethanol clearance from the amniotic fluid suggesting that this compartment acts as an ethanol reservoir [81]. In humans, the dose-response relationship between prenatal ethanol exposure and fetal effects needs to be better established. To this end, developing better methods to accurately quantify prenatal ethanol exposure is required. A method that accounts not only for dose but also for pattern and timing of exposure was recently shown to be more sensitive at detecting neurobehavioral effects of MPAE [10]. As a reference, moderate consumption was defined in that study as 70 g ethanol/week and 3-4 standard drinks/occasion (standard drink = 10 g of ethanol in Australia).

Box 2. Outstanding Issues

- Although studies have demonstrated potent effects of acute ethanol exposure on developing neurons *in vitro* [35, 82, 83], animal studies that have specifically characterized the long-lasting neurobehavioral effects of sporadic light drinking during pregnancy remain to be performed (for instance, the equivalent of 1-4 drinks/month in humans [84]).
- Pregnant women often consume ethanol at moderate doses concomitantly with other substances (e.g., nicotine and cannabis). The possibility that co-exposure to these substances modifies the effect of ethanol should be further studied [61].
- The role of genetic, epigenetic, and environmental factors in determining sensitivity of the developing brain to MPAGE should be further explored.
- Studies of the effect of MPAGE should be expanded to other brain regions. Investigators have begun to address this issue; e.g., reduced soma size of M-type neurons in the lateral geniculate nucleus (a thalamic nucleus that relays visual information) was recently demonstrated in non-human primate adult offspring (voluntary drinking during pregnancy; BAL=0.06-0.13 g/dl) [85].
- The majority of animal studies have demonstrated correlations between behavioral deficits and alterations in neuronal function. Future studies should assess whether restoration of neuronal function in specific brain regions reverses or ameliorates behavioral impairments. Studies of this nature are essential for developing an integrated view of the neurobehavioral impact of MPAGE-induced alterations in multiple neurotransmitter systems across different brain regions.
- In order to better understand the mechanism of action of ethanol, its effect on the developmental trajectory of affected cell populations should be assessed. This type of study will increase understanding of how a relatively transient developmental insult (i.e., ethanol exposure) results in alterations that persist into adulthood.
- Postmortem brain tissue from patients with FASDs is not widely available to the research community. Brain banks across the world should make a concerted effort to increase availability of these samples, allowing confirmation of key findings of animal studies with human tissue.
- In light of the results of animal experiments, human epidemiological studies should assess the effect of MPAGE on a wide range of cognitive domains across different stages of development.

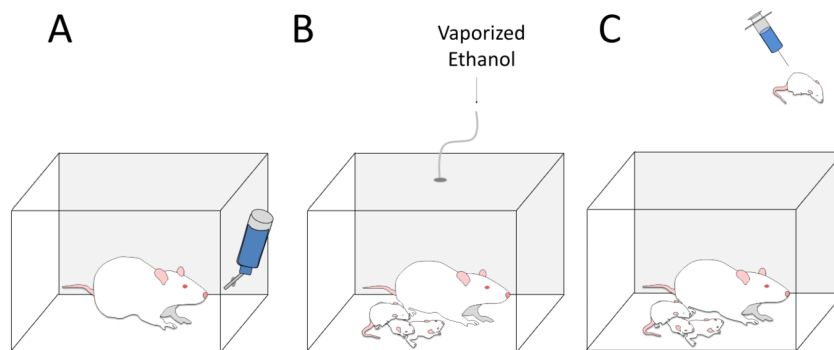


Fig 1. Schematic representation of ethanol exposure paradigms used in the studies reviewed here To model 1st and 2nd trimester ethanol exposure, pregnant rodents were exposed to moderate doses of ethanol using: A) forced (i.e., ethanol containing solutions were the only source of water and/or food [25, 46, 58]) and continuous or limited voluntary drinking paradigms [26, 29, 30]. Voluntary drinking has also been used to expose monkeys to ethanol at different stages of pregnancy [62]. B) To model human exposure during the 3rd trimester, rat pups and dams were exposed via ethanol vapor inhalation chambers [35, 55] or C) pups via intraperitoneal or subcutaneous ethanol injections [48, 64].

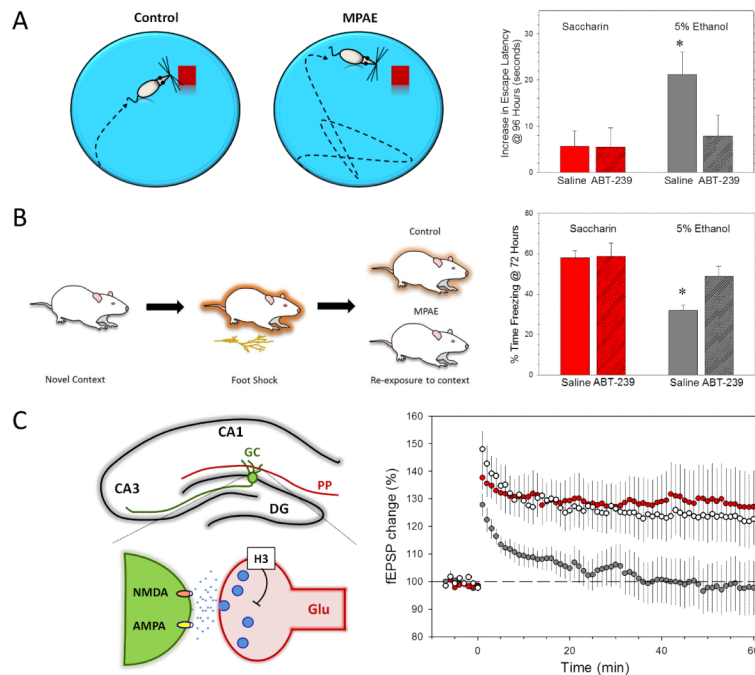


Fig 2. Prenatal exposure to moderate ethanol levels impairs hippocampal-dependent memory and plasticity

A) Left panel: schematic representation of the Morris Water Task, which measures training-induced changes in the time to find a hidden platform (escape latency) in a tub full of opaque water. Animals typically use environmental cues to locate the platform. MPAE animals typically need longer time to find the escape platform. Right panel: escape latency was significantly increased in MPAE adult rat offspring (voluntary drinking paradigm during pregnancy; 5% ethanol (v/v) plus 0.066% saccharin (v/v) in water) and this effect was reversed by the H3 receptor antagonist, ABT-239 [26]. B) Left panel: schematic representation of the contextual fear conditioning test, which measures immobility (i.e., freezing) time in rodents re-exposed to the environmental context where they received a foot shock. MPAE animals typically freeze less (i.e., fail to link the context with the shock received previously) than control animals. Right panel: freezing in a contextual fear conditioning test was reduced in MPAE adult offspring and this effect was reversed by ABT-239 [26]. C) Left panel: shown in the top panel is a schematic representation of a coronal section of the hippocampal formation showing the CA1 and CA3 hippocampal subfields, as well as the dentate gyrus (DG). A granule cell (GC) in the DG is shown in green. GCs receive glutamatergic input from the entorhinal cortex via the perforant path (PP). The lower panel illustrates a PP-GC synapse, including presynaptic H3 receptors and postsynaptic NMDA and AMPA receptors. Prenatal ethanol exposure may result in a long-lasting increase in the activity of H3 receptors, decreasing glutamate release and synaptic plasticity. Right panel: LTP recorded in the DG from urethane anesthetized rats was impaired in MPAE adult offspring (gray circles) with respect to saccharin control group offspring (red circles) and this effect was reversed by the H3 receptor blocker ABT-239 (white circles). The graph shows the change in field excitatory postsynaptic potential (fEPSP) amplitude over a 1hr period. Reprinted with permission from [26] (Panels A, B; © Wiley-Blackwell) and [27] (Panel C; © American Society for Pharmacology and Experimental Therapeutics).

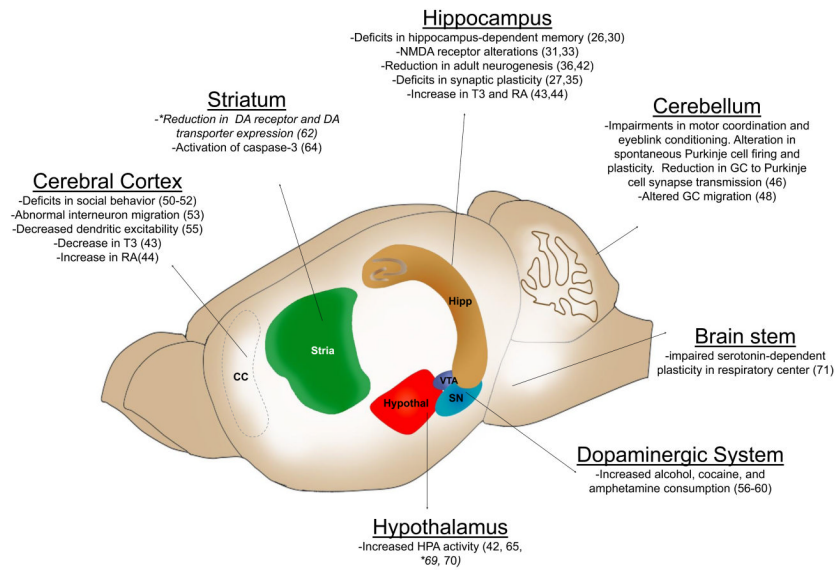


Fig 3. Schematic representation of the rodent brain indicating examples of the effect of moderate ethanol exposure in different brain regions

*For comparison, the results of studies with primates are also mentioned in *italics*.

Abbreviations: DA, dopamine; GC, granule cells; HPA, hypothalamic-pituitary-adrenal axis; RA, all-*trans* retinoic acid; SN, substantia nigra; T3, triiodothyronine; VTA, ventral tegmental area.