Low-dose prenatal alcohol exposure modulates weight gain and eliminates fractalkine expression in e14.5 mouse embryos

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Fetal Alcohol Spectrum Disorder (FASD) is caused by maternal alcohol consumption during pregnancy and often leads to long-lasting developmental symptoms, including increased microglial migration and increased release of the chemokine, fractalkine, both of which play a role in embryonic brain development. However, the effects of low-dose alcohol exposure on microglia and fractalkine embryonically are not well documented. This study addresses this gap by using the voluntary drinking paradigm, Drinking in the Dark (DiD), to expose mice to acute doses of alcohol from embryonic day 7.5 (E7.5) to E14.5. Maternal mice and embryo analyses revealed increased embryo weights and a trend of increased gestational weight gain in alcohol-exposed mice compared to water-exposed mice. After quantifying soluble fractalkine concentrations through Western Blots, results indicated decreased fractalkine in alcohol-exposed mice compared to water-exposed. Overall, our data suggest that exposure to low doses of alcohol inhibits fractalkine release, which may affect microglial function.

Abbreviations: BCM – Brain Conditioned Media; CNS – Central Nervous System; CSB – corticostriatal boundary; DiD – Drinking in the Dark; FASD – Fetal Alcohol Spectrum Disorder; RIPA – radioimmunoprecipitation assay

Keywords: DiD; FASD; microglia

Introduction

Individuals prenatally exposed to alcohol who are given the diagnosis of Fetal Alcohol Spectrum Disorder (FASD) display a wide range of neurological deficits, including physical, cognitive, and behavioral impairments. Due to alcohol's toxicity, exposure during fetal development is associated with abnormalities in various brain structures and functions (Drew et al., 2015). Although preventable, prenatal alcohol exposure impacts 87-103 newborns in the United States each day (Lupton et al., 2004). Therefore, research into the underlying mechanisms of FASD will help to develop possible therapies and interventions to enhance the quality of life for affected individuals.

FASD symptoms, including physical deformities, cognitive impairments, and behavioral deficits, are correlated with alcohol's impact on various brain structures. For example, cognitive and behavioral deficits may be due to alcohol-induced effects on certain cortical structures, thus causing the reward pathways of the brain to be significantly impaired (Prestoz et al., 2012). However, FASD symptoms can range from mild to severe in affected individuals, and differences in severity of expression depend on the quantity of alcohol an individual is prenatally exposed to and the specific gestational days of exposure (Lipinski et al., 2012). Previous research indicates that moderate

alcohol consumption (less than one drink per day) during the first trimester is correlated with impaired memory function persistent throughout adolescence (Willford et al., 2004).

While there is significant research on high-to-moderate levels of alcohol exposure, low level exposure has not been as widely investigated. Since many mothers unknowingly put their fetus at risk by engaging in mild alcohol consumption, low level alcohol exposure during pregnancy is particularly important to study to create awareness of behaviors to avoid during pregnancy, especially in the first trimester. How low dose alcohol exposure affects microglia cells, the chemokine, fractalkine, and dopaminergic axon growth is essential to study, since these cells and molecules interact and are essential components in proper neurological function (Squarzoni et al., 2014; Roberson et al., 2011).

Microglia cells are involved in the innate immune response of the central nervous system (CNS) and comprise approximately 10% of the total brain cell population (Benarroch, 2013). They exist in two different conformations: surveying or "resting" microglia, and activated microglia (Benarroch, 2013). Microglia respond to neural cell injury by signaling with immune molecules such as cytokines and chemokines (Terasaki and Schwarz., 2016). They also maintain cellular, synaptic, and myelin homeostasis during development (Benarroch, 2013), as they are present in human embryonic brains as early as 8 weeks gestation and embryonic day (E)10 in rodents (Terasaki and Schwarz, 2016). Mid-neurogenesis (E14.5) is the critical developmental point in which microglia form and associate with dopaminergic axonal tracts (Reemst et al., 2016), which recruit microglia to promote the phagocytic pruning of the axons (Squarzoni et. al, 2014).

Given microglia's neuroprotective elements and ability to produce inflammatory mediators in response to brain damage, they are essential to study in FASD models (Saito et al., 2016). Previous studies suggest that prenatal alcohol exposure induces the activation of microglia (Saito et al., 2016). Due to the fact that alcohol exposure is associated with inflammation in the brain, and microglia are the immune cells of the brain that clear inflammation, an increased amount of microglial migration and activation is expected in the presence of prenatal alcohol exposure (Terasaki and Schwarz, 2016). To study potential effects of low dose alcohol exposure on the activation and migration of microglia, it may be beneficial to conduct observation at mid-neurogenesis and to examine the role of fractalkine, which serves as a chemokine attractant for microglia (Sokolowski et al., 2014).

Fractalkine is expressed throughout the CNS and has properties that allow it to protect cells against inflammation. As a neuroprotective chemokine, fractalkine reduces neuronal death by facilitating communication between injured neurons and microglia (Roberson et al., 2011). Fractalkine recruits microglia, which contain the fractalkine receptor, CX3CR1, toward apoptotic or injured neurons, where microglia perform various immunological functions such as removal of cell debris (Sokolowski et al., 2014). In fact, many studies documented that microglial migration is dependent on the amount of soluble fractalkine present (Sokolowski et al., 2014; Squarzoni et al., 2014). Fractalkine may also be associated with neuron development by signaling microglia to migrate toward a growing neuron, where it can regulate the neuron's axon outgrowth (Squarzoni et al., 2014).

Of interest to this field is investigating microglial neuroprotective capabilities and fractalkine signaling in low dose alcohol exposure, in the absence of apoptotic conditions. This is based on the rationale that activated microglia, in response to moderate prenatal alcohol exposure, potentially provide neuroprotection via chemokine signaling (Saito et al., 2016). Furthermore, moderate alcohol exposure in the fetal brain causes increased chemokine expression, further suggesting that alcohol exposure leads to increased immunological signaling in the brains of exposed individuals (Terasaki and Schwarz, 2016). Previous research has indicated that high levels of alcohol exposure cause an increase in microglial migration as well as an increase in the expression of fractalkine (Roberson et al., 2011). A study by Roberson and colleagues found significantly higher levels of fractalkine in their alcohol-exposed mice just six hours after

alcohol exposure (Roberson et al., 2011). While low levels of alcohol exposure do not induce neuronal apoptosis (Rodriguez et al., 2015), there still may be an increased release of chemokine signaling and subsequent microglial migration and activation (Pont-Lezica et al., 2014). However, it remains unknown if these effects persist and microglia continue to migrate toward dopaminergic neurons in response to lower levels of prenatal alcohol exposure

Dopaminergic axons are of interest because they develop during mid-neurogenesis, the same time in which embryonic microglia play a role in development, and are known to physically interact with microglia (Squarzoni et al., 2014). These axons must follow specific steps to ensure appropriate embryonic axon guidance, requiring many signals to regulate migration (Prestoz et al., 2012). Several reward pathways within the brain involve dopaminergic neurons extending from the ventral tegmental area and branching out to the prefrontal cortex (Ikemoto and Panksepp, 1999; Wise and Rompre, 1989). If the axons of dopaminergic neurons fail to successfully grow toward the ideal brain regions during early development, this pathway will not innervate properly and reward function will thus be impaired. Notably, when a lesion affects the molecules involved in dopaminergic axon guidance, axon tracts cannot regenerate, causing dopaminergic axon death (Prestoz et al., 2012). This indicates the importance of certain molecules and cells in normal dopaminergic axon functions.

As previously suggested, microglia may play a role in dopaminergic axon guidance through phagocytosis of the axons. Specifically, at E14.5, dopaminergic axons extend toward the corticostriatal boundary (CSB), where they are in close proximity to microglia (Squarzoni et al., 2014). Importantly, an increase or decrease in microglial density alters axon extensions toward the CSB. A model of depleted microglia causes increased dopaminergic axon extensions closer to the CSB, while an enhanced microglial model causes reduced extensions and restricted growth further from the CSB. The resulting abundance of immunological signaling molecules would thus induce a neuroinflammatory state that may cause neuronal dysfunction and impaired axon guidance (Squarzoni et al., 2014). Therefore, by examining levels of microglial migration, the resulting effects on dopaminergic axons during development can be studied.

While substantial evidence exists on the relationship between microglia and dopaminergic axons, little is known about alcohol's specific effects on dopaminergic axon development. Even though other neurotransmitter systems may be impacted by fetal alcohol exposure, dopaminergic axons are of concern due to the relationship between the axons and microglia, and the established effects of alcohol on microglia. While many of the characteristic symptoms of FASD are also the result of changes in the normal development of the dopaminergic axons (Schneider et al., 2005), scientists have not directly explored this correlation, especially in a low-dose model. To date, researchers can infer alcohol's effect on dopaminergic axons through its effect on microglia, but this inference is not strong enough to pinpoint altered axon guidance as the cause of FASD symptoms.

The purpose of this study is to explore the relationship between fractalkine levels, microglial migration, and dopaminergic axon guidance following acute prenatal alcohol exposure through the voluntary Drinking in the Dark (DiD) paradigm, in an attempt to identify mechanistic causes of the less pronounced symptoms of FASD. Based on previous studies on the relationship between alcohol and fractalkine, we hypothesized that alcohol exposure in E14.5 mice would cause increased fractalkine protein concentrations in the alcohol-exposed condition compared to the control, water-exposed condition.

Materials and Methods

Animals and Animal Facility

Pregnant outbred Swiss Webster mice were timed by Charles River (Wilmington, MA) so that they would be shipped to the animal facility at Ursinus College on embryonic day (E) 1.5. Dams were weighed upon arrival and individually housed in standard shoebox mouse cages. Dams were randomly assigned as either tap water exposed (n=6) or alcohol-exposed (n=7). Food was freely available to mice at all

times. Water was freely available for 22 hours each day. The mouse facility was maintained on a reverse 12-hour light-dark cycle with lights out at 1:30pm. The temperature and humidity of the mouse facility was maintained at 21±1℃ and 60 ±5%, respectively. All procedures were approved by Ursinus College's Institutional Animal Care and Use Committee (IACUC, permit #A4347-01) and are in accordance with National Institutes of Health (NIH) guidelines on the care and use of animals.

Drinking in the Dark (DiD) Exposure Paradigm

For this study we used the DiD exposure paradigm described by Boehm et al. (2008). Beginning 3 hours after lights out (at 4:30pm) each day, water bottles were replaced with a sipper tube of either tap water (water-exposed) or 5% (v/v) ethanol diluted in tap water (alcohol-exposed). After 2 hours, sipper tubes were removed and water bottles were replaced. Food was freely available throughout the procedure. DiD was performed from E7.5 until dams were euthanized on E14.5. Each day each sipper tube was weighed at the start and end of DiD. Dam weights on E7.5 and E14.5 were used to determine maternal liquid consumption and alcohol intake. Maternal liquid consumption was calculated as grams of solution consumed divided by body weight in kilograms. Alcohol intake was calculated by multiplying maternal liquid consumption for alcohol drinking dams by the percentage of the solution (5%) then the density of ethanol (0.789g/ml).

On the day of euthanasia, embryos were harvested by caesarean section of pregnant dams. Litter size was determined by counting the number of resultant embryos. The average embryo weight was calculated by weighing the entire litter and then dividing by the number of embryos. Embryos were either immersion fixed in 4% paraformaldehyde (diluted in phosphate buffered saline, Sigma #P3813 St. Louis, MO, from a 20% v/v solution, Electron Microscopy Sciences #157-SP Hatfield, PA) for future studies or brains were removed to generate samples for brain conditioned media (BCM) and Western blotting.

Fractalkine Western Blot of embryos

Brain conditioned media was generated by incubating 16 cortical hemispheres dissected from E14.5 embryo brains in 3 ml DMEM for 2 hours on ice with periodic agitation. In the case of RIPA lysed samples, each E14.5 mouse brains were lysed in 500 µL RIPA (Thermo Scientific #P189900 Waltham, MA). An 18-gauge syringe was used to homogenize tissue. Ten µL of BCM or lysate was used to determine protein content using BCA Protein Assay according to manufacturer instructions (Pierce #23227 Rockford, IL). BCM and lysates were frozen at -80ºC until they were sent to RayBiotech (Norcross, GA) for analysis via the Simple Western testing service. All samples were loaded as 0.5 mg/mL. Fractalkine was detected with a goat anti-fractalkine antibody provided by RayBiotech; tubulin was detected with a mouse anti-alpha-tubulin antibody provided by RayBiotech.

Results

Alcohol-exposure affected embryo weight and gestational weight gain

Analyses of maternal mice and embryos were taken during and after the DiD paradigm to determine the impact of low-dose voluntary alcohol exposure. Results indicated that alcohol exposed embryos weighed significantly more than water-exposed embryos (Table 1; $p=0.03$). Additionally, there was a trend of increased weight gain in alcohol-consuming mothers, which was not observed as prominently in water-consuming mothers (Table 1; $p=0.05$). However, there was no significant difference in fluid consumption between water-consuming and alcohol-consuming mothers (Table 1; $p=0.44$), or in litter size of alcohol-exposed mice and water-exposed mice on E14.5 (Table 1; $p=0.94$). The average alcohol consumption from E7.5 to E13.5 was 1.14±0.27 g/kg (Table 1).

Alcohol exposure impacted fractalkine release in brain conditioned media, but not in RIPA samples

In order to determine soluble fractalkine protein concentrations in alcohol- and water-exposed media conditions, Western Blot analyses of brain conditioned media (BCM) and lysed brains in radioimmunoprecipitation assay (RIPA) buffer samples were performed (Figure 1A). Results from the BCM samples indicated that alcohol-exposed BCM contained a miniscule amount of soluble fractalkine, while water-exposed BCM contained visibly greater amounts of protein, as seen from the width and darkness of the bands (Figure 1B). The Western Blot of RIPA samples illustrated the presence of soluble fractalkine for all water-exposed RIPA samples, and all but one alcohol-exposed RIPA sample (Figure 1C). All fractalkine bands were visible at the expected molecular weight of soluble fractalkine, ~120kDa (Figure 1B-C), and the alpha-Tubulin loading control showed bands at the expected molecular weight of 50kDa (Figure 1C). Based on a t-test, no significant differences were found between alcohol- and water-exposed RIPA samples for the ratio of soluble fractalkine to alpha-Tubulin, as determined from areas under chemiluminescence curves (Figure 1D; p=0.11). Although no statistical analyses could be conducted on alcohol- and water-exposed BCM samples (since protein concentrations of soluble fractalkine were negligible), the large difference in fractalkine between the two exposure conditions led to the presumption that there was a significant effect of alcohol on fractalkine release in BCM (Figure 1B).

Table 1. Analysis of maternal and embryo weight, size, and fluid intake between exposure-conditions indicates significant increase in embryo weight for ethanol-exposed mice. Data show no significant differences between control (n=6) and ethanol-exposed (n=7) conditions for gestational weight gain (control: n=6; ethanol: n=7; p=0.05), maternal fluid consumption (control: n=3; ethanol: n=3; p=0.44), and litter size (control: n=6; ethanol: n=7; p=0.94), but show a significant difference for embryo weight (control: n=5; ethanol: n=7; p=0.03). Averages and standard deviations were determined for each mouse over designated time ranges when applicable, and then calculated for control- and ethanol-exposed groups. P-values were determined from a two-tailed t-test with unequal variance, and * denotes statistical significance.

	Control (H_2O)	Prenatal Alcohol Exposure (5% ethanol)	P-value (two-tailed t-test)
Gestational Weight Gain from E7.5 to E14.5 (g)	13.24 ± 0.91	15.06 ± 1.76	0.05
Maternal Fluid Consumption from E7.5 to E13.5 (g/kg body weight)	43.19 ± 26.15	28.88 ± 15.97	0.44
Maternal Ethanol Intake from E7.5 to E13.5 (g EtOH/kg body weight)	N/A	1.14 ± 0.27	N/A
Litter Size on E14.5 (number of embryos)	13.5 ± 1.80	13.57 ± 1.18	0.94
Embryo Weight on E14.5 (g)	0.33 ± 0.04	0.41 ± 0.05	0.03*

Discussion

This study used a mouse model to explore the effect of acute alcohol exposure on maternal and embryonic characteristics, as well as fractalkine release, in order to model cellular and molecular effects of mild FASD in humans. After completion of the DiD paradigm, results showed that prenatal alcohol exposure caused increased embryo weights and a trend of increased gestational weight gain, but had no effect on litter size or maternal fluid consumption (Table 1). Additionally, results indicated that alcohol-exposed BCM expressed less fractalkine than water-exposed BCM, but that alcohol had no significant effect on fractalkine concentrations in

RIPA samples (Figure 1). These results suggest that microglial phagocytosis of dopaminergic axons may be reduced in the alcohol-exposure condition and provide implications into cellular and molecular causes of FASD from a low alcohol exposure paradigm.

DiD paradigm has minimal effects on maternal and embryo characteristics

Overall, alcohol exposure through the DiD paradigm only significantly impacted embryo weights and showed a trend towards affecting gestational weight gain. Alcohol

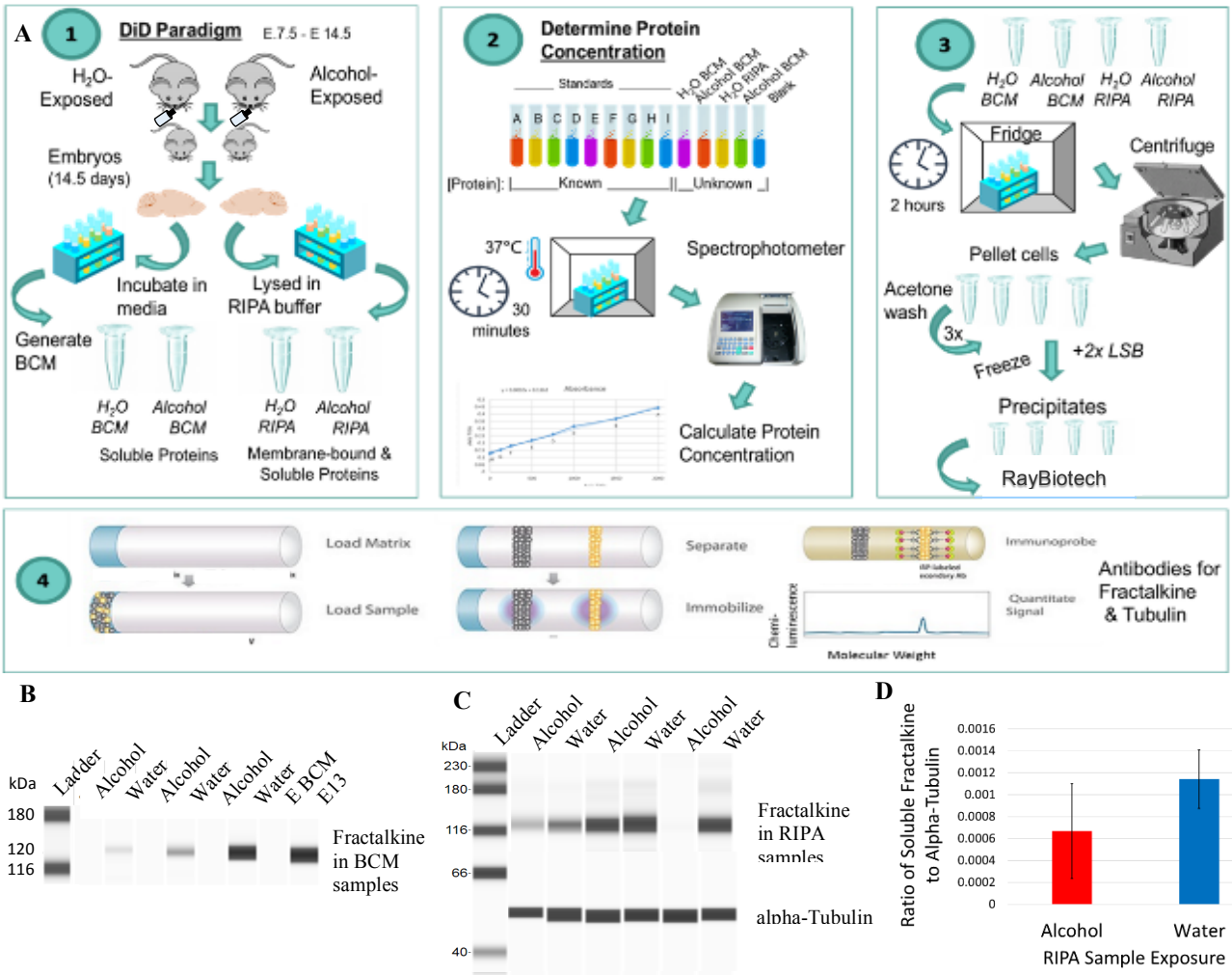

Figure 1. Ethanol exposure decreases fractalkine expression in brain conditioned media, but does not significantly affect RIPA samples. (A) Methodology performed for Western Blot analysis. (B) Western Blot of fractalkine protein from alcohol- and water-exposed brain conditioned media, with bands for water-exposed conditions visible at ~120 kDa for soluble fractalkine. (C) Western Blot of fractalkine protein (top bands) and alpha-Tubulin loading control (bottom bands) for alcohol- and water-exposed lysed brains in RIPA buffer, with bands for soluble fractalkine visible at ~120 kDa and bands for alpha-Tubulin visible at ~50kDa. (D) Quantification of ratio of soluble fractalkine to alpha-Tubulin from RIPA samples, analyzed through areas in chemiluminescence curves. No significant differences were found between alcohol and water conditions (p=0.11). Error bars were determined from standard deviations, and a two-tailed t-test with unequal variance was conducted to determine significance between treatment exposures for RIPA samples.

exposure led to significantly greater embryo weights at E14.5 than the control group (Table 1; p=0.03), which is consistent with Cuzon et al. (2008). They used an acute alcohol-exposure model (2% alcohol) with inbred mice and found a significant increase in embryo brain and body weight after alcohol-exposure (Cuzon et al., 2008). However, our findings contrast those by Chi et al. (2016) and El Shawa et al. (2013), which showed no such difference in pup weight, and reported significantly decreased pup weight for the alcohol-exposed condition at birth, respectively. Importantly, these studies used low and moderate voluntary exposure paradigms with outbred mouse strains, similar to the current study, so these contradicting results could be due to the amount of alcohol the mice in each study actually consumed.

Additionally, these findings showed a trend towards increased gestational weights for alcohol-exposed mice (Table 1; p=0.05). Boehm et al. (2008) found no significant differences in gestational weight gain between alcohol and control mice when adjusting for initial weights of the mice, but they found a trend of decreased gestational weights for alcohol-exposed mice. The current study also found no significant differences in maternal fluid consumption or litter size between alcohol- and water-exposed mice (Table 1), which contradicts the decreases in these parameters in Boehm et al. (2008). One potential reason for these contradicting results is that Boehm et al. used binge doses of alcohol (20% alcohol), and even though the drinking was voluntary, higher doses could have a more substantial impact on the embryos (Boehm et al., 2008). Importantly, the mice in the current study only drank on average 1.14±0.27 g alcohol/kg body weight (Table 1), which may not be a high enough dose to cause any effect. The non-significant data observed in the current study, potentially due to a lack of alcohol consumption, could explain the seemingly contradictory levels of fractalkine release that were observed in the alcohol-exposed media conditions.

Alcohol-exposure hinders fractalkine release from brain conditioned media

Previous research focusing on high alcohol exposure paradigms showed that fractalkine signaling increases due to the release of chemokines from apoptotic neurons (Sokolowski et al., 2014). This study's results indicated that alcohol-exposed BCM contained significantly less soluble fractalkine than water-exposed BCM, and illustrated no significant differences between treatment conditions for RIPA samples (Figure 1). Even though we could not perform statistical analyses for BCM samples, a significant difference between conditions can be assumed due to the negligible amount of fractalkine present in the alcohol-exposed condition. Since this study used low-dose alcohol exposure, it is assumed that no apoptosis occurred in our model (Rodriguez et al., 2015). These results suggests that low-doses of alcohol block soluble fractalkine release and that the cause of increased fractalkine release is apoptosis due to high alcohol consumption, not the introduction of alcohol in general. This contradicts the current study's hypothesis, as well as Terasaki and Schwarz (2016), which observed increased fractalkine signaling with a low exposure paradigm in rats. The timing of alcohol exposure for their study, E10-E16 (Terasaki and Schwarz, 2016), was quite similar to that of the current study, so it raises questions as to why these data do not show a similar trend.

However, Rodriguez and colleagues' study on alcohol consumption and cardiovascular disease suggests that low doses of alcohol are associated with decreased levels of apoptosis (Rodriguez et al., 2015). Since fractalkine is released from apoptotic cells, this decreased apoptosis could justify the low fractalkine concentrations in the alcohol-exposed condition, if in fact low alcohol consumption affects the brain in the same manner as in the heart. Interestingly, Roberson and colleagues suggest that increased fractalkine may play a harmful role in causing microglial dysfunction (Roberson et al., 2015), implying that low levels of fractalkine, corresponding to low levels of apoptosis, may be a protective mechanism. Overall, these findings suggest that low doses of alcohol hinder soluble fractalkine release, and this factor is especially important when observing the relationship between fractalkine signaling and microglial migration.

Alcohol exposure may affect dopaminergic axon extensions

Previous studies established the connection between microglia and dopaminergic axons; microglia regulate axon growth towards the CSB through phagocytosis of the axons (Squarzoni et al., 2014). Therefore, increased density of microglia near the axons should lead to more phagocytosis, and vice-versa. Squarzoni et al. found increased dopaminergic axon extensions in a depleted microglia model with a knocked-out fractalkine receptor (CX3CR1), where microglia lose the ability to sense fractalkine signals (Squarzoni et al., 2014). This inability to sense fractalkine is comparable to the decrease in soluble fractalkine release observed in the alcohol-exposed conditions (Figure 1B), since in both cases, microglia would not migrate towards the chemokine signal. This decreased migration towards fractalkine-releasing neurons implies that with alcohol exposure, dopaminergic axon extensions should increase closer to the CSB due to decreased phagocytosis. While the mechanism of axon outgrowth is not known, it may be interesting to further research the role of ephrin-A5, a molecule with an expression gradient that correlates with dopaminergic axon growth during mid-neurogenesis (Prestoz et al., 2012), to determine if low-dose alcohol exposure impacts its function and axon outgrowth simultaneously. While dopamine axons may be altered in FASD patients, most studies focused on FASD symptoms in a high exposure paradigm. It is also possible that low alcohol doses alter axon outgrowth, and this could explain why acute alcohol exposure produces less pronounced symptoms (Wierzba-Bobrowicz et al., 2003).

Future directions and questions

To achieve more statistically significant results in the future, implementing a different alcohol-exposure paradigm to better monitor alcohol intake, such as injections, would be a preliminary step. While the DiD paradigm is ideal for mimicking human's varied alcohol consumption, it is limited because it leaves the possibility that some mice in the alcohol exposure group will not drink. In the current study, all alcohol-exposed dams drank, but some drank very little. It may be possible to still mimic varied alcohol consumption through injections by using different groups of alcohol-exposed mice with different doses. Researchers could also begin exposing the mice to alcohol at an earlier time point than E7.5 to see if earlier exposure during pregnancy causes more significant impacts on fractalkine release and microglia. Further research could quantify microglial migration towards alcohol- and water-exposed brain conditioned media, as well as explore dopaminergic axons in E14.5 mouse embryos. Additional studies could quantify a rough estimate of the amount of microglial migration required for significant pruning of axons, and the effect of acute alcohol exposure on this process. Performing behavioral and developmental milestone tests on mice that underwent a mild exposure paradigm would be useful in determining if they exhibit any significant social behaviors that are characteristic of FASD.

This study brings forth additional questions regarding the relationship between alcohol, fractalkine, and microglia. Whether alcohol increases or decreases fractalkine expression in a dose- or time-dependent manner still needs to be explored, as does the role of alcohol and fractalkine in altering microglial migration. Ultimately, these findings put in question whether acute doses of alcohol during pregnancy negatively impact the fetus, or if higher doses are needed to cause FASD in exposed individuals. Exploring this question is critical in attempting to prevent pregnant women from engaging in mild alcohol consumption and potentially putting their fetus at risk, and to determine the minimum amount of alcohol that induces symptoms of FASD.

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