Abstract

Prenatal alcohol exposure (PAE) can result in a range of mental disabilities and physical defects. Because adults affected by PAE demonstrate increased susceptibility to alcohol dependence, it was hypothesized that PAE leads to the loss of inhibitory GABAergic interneurons in corticostriatal circuits that regulate reward perception. Offspring of mice that experienced acute ethanol exposure on gestation day 7 (GD 7) were used as an animal model. Intracranial self-stimulation studies showed a marked difference in threshold for non-exposed controls and PAE mice during the first 15 minutes following morphine injection, but no significant difference was shown with alcohol or cocaine. Immunohistochemistry against parvalbumin was performed to visualize the GABAergic interneurons of the CNS in the nucleus accumbens, medial prefrontal cortex, dorsal striatum, and medial septum. A preliminary count of the interneurons in the nucleus accumbens showed no difference between control and PAE mice. In the future, further quantification of GABAergic interneurons in all four regions of interest may conclude that maternal ethanol ingestion on GD 7 causes significant damage to the normal distribution of inhibitory interneurons in CNS structures associated with reward perception.

Introduction

Fetal alcohol syndrome (FAS) results from severe ethanol exposure during fetal development and is characterized by stunted fetal growth, distinctive craniofacial abnormalities, and damage to the central nervous system (CNS) that can lead to a spectrum of intellectual, psychological, and social disabilities. Fetal alcohol spectrum disorders (FASD), an umbrella term that encompasses FAS and all other developmental disabilities associated with prenatal alcohol
exposure (PAE), represent the most common non-inherited cause of birth defects in the western world (Clarke and Gibbard, 2003). The efficacy of animal models in the study of PAE has been demonstrated (eg. Driscoll et al., 1990; Wilson, 2011) and subsequent studies have employed diverse methodologies to better understand the effects of FAS in humans.

Studies in PAE have been successful in utilizing neuroimaging techniques such as magnetic resonance imaging (MRI) to observe anatomical malformations of brain structures in both humans and animal models (Bookstein et al., 2006; Lipinski et al., 2011). It was concluded that anatomical malformations of facial features and brain structures during fetal development are variable and stage-dependent in PAE mice. In both humans and mice, the malformations observed depended on the day of gestation ethanol exposure occurred. Furthermore, magnetic resonance imaging of mouse models for PAE correlated to imaging of humans affected by PAE (O’Leary-Moore et al., 2011).

Quantifiable evidence of damage to the central nervous system (CNS) due to PAE is lacking. Based on previous observations of increased alcohol dependence in adults affected by PAE (eg. Yates, 1998), we hypothesized that maternal ethanol ingestion during gestation leads to neuronal loss in corticostriatal circuits involved in regulating reward perception and that this damage may enhance both the positive and negative effects of alcohol and other drugs. Preliminary findings utilizing intracranial self-stimulation (ICSS) as a means of delivering direct stimulation to the brain’s reward circuitry showed no significant differences in brain stimulation reward (BSR) between PAE mice and non-exposed controls following acute exposure to ethanol (Figure 2A). This result did not support the initial hypothesis that PAE mice would exhibit increased alcohol intake and alcohol reward relative to control mice. Similarly, no significant difference was seen following the administration of cocaine although it was hypothesized that
alcohol and drugs such as cocaine would have an enhanced effect on reward perception in PAE mice relative to the control (Figure 2B). However, a significant difference was found between the two groups in the potentiating effects of morphine (Figure 2C). PAE mice exhibited greater sensitivity to morphine and experienced a greater potentiation effect in comparison to non-exposed controls. This result supports our hypothesis, but past studies have shown that PAE’s effect on pituitary-adrenal function can lead to enhanced responses to footshock stress and morphine amongst other stress related responses (Nelson et al., 1986). Therefore, further studies are required to analyze whether a change in reward perception was involved in the dissimilar response to morphine that was observed in the PAE and control mice.

Investigation of GABAergic interneuronal loss in structures of the brain underlying reward perception was the next step in understanding the effects of PAE on the CNS. GABA is the chief inhibitory neurotransmitter in the CNS. Studies have demonstrated that imbalances in neuroexcitatory and neuroinhibitory amino acids, such as glutamate and GABA respectively, can result from long-term ethanol exposure and are responsible for behavioral susceptibility during ethanol withdraw (De Witte, 2004). Because insufficient expression of GABA in the CNS can lead to an imbalance in inhibition and excitation, these conclusions support the hypothesis that loss of GABAergic interneurons due to PAE may lead to increased alcohol dependency.

The process of performing a morphological analysis of GABAergic interneurons in the nucleus accumbens, medial prefrontal cortex, dorsal striatum, and medial septum of both PAE and non-exposed control mice was started using immunohistochemistry to visualize the interneurons of interest and was followed by a manual count. Design-based stereology will be utilized to quantify the interneurons in an unbiased manner in future counts. Immunohistochemistry against parvalbumin, a calcium-binding globular protein that is prevalent
in GABAergic interneurons, required a new protocol for effective staining due to a lower density of inhibitory interneurons in the nucleus accumbens relative to the other three regions of interest. Previous work done by the lab has achieved proper staining of the medial prefrontal cortex, dorsal striatum, and medial septum. The optimal protocol for staining in the nucleus accumbens was determined by testing multiple primary antibody ratios and incubation periods and assessing them with brightfield microscopy (Table 1 and 2).

In the near future, the samples that have undergone staining for parvalbumin will be assessed using design-based stereology that allows for systematic random sampling (Figure 3a). The availability of design-based stereology in quantitative morphology of the CNS has allowed for neuronal counting in entire brain structures of interest without bias due to the size, shape, spatial orientation, or spatial distribution of cells (Schmitz and Hof, 2005). Quantifying the extent of neuronal loss or maldistribution in FAS mice may indicate the circuits and routes of neuronal migration damaged by PAE to provide insight into the origin of defects in reward perception and motivated behavior observed in adults affected by FAS.
Figure 1. The craniofacial abnormalities of FAS individuals correlated with magnetic resonance imaging of malformations in facial features and brain structures of PAE mice exposed on gestation day 7 (GD7) and gestation day 8.5 (GD8.5). The malformations in the mouse models are stage dependent and correlate to those seen in humans (O’Leary-Moore et al., 2011).
Figure 2A. No difference was shown in threshold or max response between PAE and non-exposed control mice following administration of alcohol.
Figure 2B. No difference was shown in threshold or max response between PAE and non-exposed control mice following administration of cocaine.

Figure 2C. A significant difference was shown in threshold between PAE and non-exposed control mice during the first 15 minutes following administration of morphine. PAE mice experienced a marked attenuation of ICSS not observed in the control mice.

Figure 3a. The systematic-random sampling made possible by design-based stereology to yield an unbiased count of interneurons of the mouse nucleus accumbens (Courtesy of Malanga, C. J.).

Figure 3b. An unbiased counting frame generated by the stereological probe (Courtesy of Malanga, C. J.)
Materials and Methods

Mice:

The investigation utilized a mouse model of FAS produced by C57BL/6J dams given acute ethanol exposure (X mg/kg, p.o.) on GD 7. The offspring of these dams were grown to young adulthood (PXX) prior to anesthetization and transcardial perfusion with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde. A non-exposed control group given no ethanol exposure during gestation was also used. All work leading up to the fixation of the brains in 4% paraformaldehyde and PBS was performed by other members of the lab.

Immunohistochemistry:

Histology was performed using coronal slices on a sliding microtome to acquire an exhaustive sample of 50 μm thick sections that encompass the entirety of all 4 brain regions of interest: the nucleus accumbens (NAc), medial prefrontal cortex (mPFC), dorsal striatum, and medial septum. These sections were placed in cryoprotectant (1.0% w/v polyvinylpyrrolidione, 30% w/v sucrose, and 30% v/v ethylene glycol in 0.1 M PBS) and stored at 0°C until use. Sections representative of entire regions of interest were removed from cryoprotectant and rinsed with 0.1 M PBS before undergoing free-floating immunohistochemistry against parvalbumin. The sections were treated with 0.05% H₂O₂ followed by a 0.5% triton with 3.0% normal goat serum solution before incubation with goat anti-parvalbumin primary antibody of varying concentrations (Table 1 and 2). Using two sets of samples, trials 1 and 2 were incubated in
primary antibody for 72 hours and 48 hours respectively. In both trials, 2 sections were stained in 2 separate wells at each primary antibody concentration. All samples were incubated in a rabbit anti-goat secondary antibody with a 1:250 concentration for 4 hours and visualized with a DAB peroxidase substrate kit. The sections were mounted on gelatin-subbed slides to be counterstained and coverslipped prior to assessment with light microscopy.

*Nissl Counterstain:*

A Nissl counterstain was performed on half of the sections from the second immunohistochemistry trial against parvalbumin. The protocol for Nissl counterstaining will be tested and modified in future immunohistochemistry trials meant to confirm the concentrations of primary antibody to be used for optimal visualization of parvalbumin in each of the brain structures of interest.

*Manual Counts:*

A preliminary count of interneurons in the nucleus accumbens of PAE and non-exposed control mice was performed with brightfield microscopy and an outline of the region of interest from a mouse brain atlas.

*Design-based Stereology:*
In the near future, a quantitative analysis of GABAergic interneurons in the nucleus accumbens, medial prefrontal cortex, dorsal striatum, and medial septum of the PAE and control brain sections will be performed with design-based stereology. The optimal counting frame area, dissector height, and guar frame width for stereological analysis of each brain region will be determined through practice trials prior to counting (Figure 3B).

**Results:**

Sections from trials 1 and 2 of immunohistochemistry against parvalbumin were incubated in goat anti-parvalbumin primary antibody for 72 hours and 48 hours respectively. The 1:1500 concentration was used as a control because this ratio was successful in immunostaining all regions of interest other than the NAc in past trials. Regardless of the concentration of primary antibody used, trial 1 resulted in sections that were too dark to accurately quantify the number of GABAergic interneurons in neither the NAc nor any of the other three structures of interest (Table 1). In contrast, trial 2 resulted in variable success with staining of inhibitory interneurons in the NAc depending on the concentration of primary antibody used. Sections that underwent incubation in a 1:690 concentration of primary antibody in trial 2 yielded the best staining for visualizing interneurons of the NAc. In trial 3, the concentrations of primary antibody used in trial 2 were repeated to better determine an intermediate protocol that visualizes interneurons in all 4 regions of interest simultaneously. A 1:1230 concentration of primary antibody was selected as the optimal protocol for our investigation (table 2). The selected protocol showed adequate staining of GABAergic
interneurons in the nucleus accumbens without over-staining in other regions of greater interneuron density (Figure 4).

Nissl counterstaining with cresyl violet was performed on 1 of the sections at each primary antibody concentration in trial 2. Only patchy staining was observed in most sections and those that were mounted towards the end of the slide showed no sign of Nissl staining (Table 2). There were insufficient amounts of Nissl staining to determine whether the white or dark matter of the sections was more heavily stained. Areas of the sections that were Nissl stained were not indicative of increased staining in any specific biological structures of the brain. However, some counterstained areas demonstrated success in providing contrast to better visualize the parvalbumin immunostaining when compared to the non-counterstained sections from trial 2 that were incubated at the same concentration of primary antibody.

Manual counts of interneurons in the NAc of the PAE and non-exposed mice showed no significant difference between the two groups in the number of interneurons present in the NAc (Figure 5). This result is contrary to our hypothesis because we expected to observe a lower number of interneurons in structures of the brain associated with reward perception in the PAE mice due to damage to the CNS during fetal development. However, a conclusion cannot be reached until the remaining brains are accounted for and a more reliable method of quantification is used to minimize sources of bias during counting. In the future, the GABAergic interneurons of the immunostained sections from both GD 7 PAE mice and non-exposed controls will be quantified using design-based stereology in the four regions of interest. Estimates of the total number of inhibitory interneurons in the nucleus accumbens, medial prefrontal cortex, dorsal striatum, and medial septum will be reported using the optical fractionator method with considerations for average section shrinkage.
Table 1. Immunohistochemistry trial 1 for determining the optimal goat anti-parv primary antibody concentration demonstrated that an incubation time of 72 hours yields overly dark staining.

Table 2. The second immunohistochemistry trial showed variable staining in the nucleus accumbens depending on the primary antibody concentration. A 1:690 concentration with 48 hours of incubation time (highlighted) yielded the best results for staining in the nucleus accumbens. A 1:1230 concentration was selected as optimal for staining in all 4 regions of interest. Counterstaining of Nissl bodies was patchy in most sections and not present in two.
**Figure 4.** Staining in the nucleus accumbens and surrounding structures using a 1:1230 concentration of primary antibody. The interneurons are easily visualized and there is no evidence of over-staining in the surrounding areas.
Figure 5. A preliminary count of GABAergic interneurons in the NAc of PAE and non-exposed control mice (n=2). There was no significant difference between the two groups thus far.

Discussion

At this point in the study, proper protocols for immunostaining of inhibitory GABAergic interneurons of the NAc, mPFC, dorsal striatum, and medial septum were established. The immunohistochemistry protocol for the latter 3 structures was determined by the work of previous lab members. Because parvalbumin immunostaining was not present in the NAc of these past trials, they demonstrated that a separate protocol was necessary. It was concluded that the NAc had a lower density of GABAergic interneurons relative to the other regions of interest. A higher primary antibody concentration, a longer incubation time with the primary antibody, or some combination of the two was necessary to properly immunostain the inhibitory interneurons in the NAc.

The three trials used to determine a protocol suitable for staining in the nucleus accumbens led to several conclusions regarding immunohistochemistry against parvalbumin. The failure of trial 1 in properly staining the GABAergic interneurons of any of the regions of interest demonstrated the significant effect that primary antibody incubation time has on immunostaining. Specifically, it was concluded that increasing incubation time leads to a decrease in specific binding by the primary antibody. Once all of the parvalbumin was bound, the remaining antibody eventually bound to other areas of the mouse brain and yielded sections that were too darkly stained to quantify the inhibitory interneurons of interest.
In contrast to sections from trial 1, trial 2 demonstrated variable staining of GABAergic interneurons depending on what concentration of primary antibody was used. As originally predicted, trial 2 showed that increasing the primary antibody concentration increases staining for parvalbumin. However, the sections from trial 2 with the two highest concentrations (1:840 and 1:300) demonstrated that increasing the primary antibody concentration also increases the prevalence of non-specific binding. It was concluded that using an overly high concentration causes excess primary antibody to bind to non-specific targets in the brain after all of the parvalbumin has been bound.

Immunohistochemistry trial 3 showed that an intermediate protocol between the concentrations of 1:1500 (good staining in mPFC, medial septum, and dorsal striatum) and 1:690 (good staining in NAc) could be used to stain for all four regions of interest on the same brain section. At a primary antibody concentration of 1:1230, interneurons in all structures of interest were properly stained. This concentration was utilized as the optimal protocol for our investigation.

The group of sections from trial 2 that underwent counterstaining led to several conclusions on how to improve the protocol for Nissl staining using cresyl violet. The lack of Nissl body staining in the sections suggests that the slides were treated with the acetic acid solution for too long following staining with cresyl violet. Interestingly, the sections mounted towards the end of the slide that made contact with the counterstaining solutions first exhibited much less Nissl staining relative to those that were mounted towards the opposite end. It was concluded that the submergence of slides in wells during Nissl staining must be done using quick dipping motions to minimize disparities in the length of time that different sections have contact with the counterstaining solutions. Slow dipping motions caused sections that are mounted
towards the end of the slide that contacts the solutions first to be submerged for longer. The results of the counterstain trial demonstrated that this difference in time can result in significant differences in Nissl staining.

The manual count of GABAergic interneurons in the NAc of the PAE and non-exposed control mice have shown no significant difference thus far. However, the quantification of interneurons in the mPFC, medial septum, and dorsal striatum has yet to be done, and further counting in the NAc is also necessary. Therefore, it is too early to make any conclusions about our hypothesis that PAE causes damage to interneurons in structures of the brain associated with reward perception. In the future, the quantification of GABAergic interneurons in the brain regions of interest will be performed using design-based stereology. Another future goal may be to study PAE’s effect on the adrenal-pituitary response and whether it has any connection to a greater propensity for alcohol and drug addiction.

**Literature Cited**


Resonance-based imaging in animal models of Fetal Alcohol Spectrum Disorder.

Neuropsychology Review 21, 167-185.


Alcoholism, Clinical and Experimental Research 22, 914-920.