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Regional patterns of alcohol-induced neuronal loss depend on genetics: Implications for fetal alcohol spectrum disorder

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Abstract

Background: Alcohol-exposure during pregnancy can kill developing neurons and lead to fetal alcohol spectrum disorder (FASD). However, affected individuals differ in their regional patterns of alcohol-induced neuropathology. Because neuroprotective genes are expressed in spatially selective ways, their mutation could increase the vulnerability of some brain regions, but not others, to alcohol teratogenicity. The objective of this study was to determine whether a null mutation of neuronal nitric oxide synthase (nNOS) can increase the vulnerability of some brain regions, but not others, to alcohol-induced neuronal losses.

Methods: Immunohistochemistry identified brain regions in which nNOS is present or absent throughout postnatal development. Mice genetically deficient for nNOS (nNOS^{-/-}) and wild type controls received alcohol (0.0, 2.2, or 4.4 mg/g/day) over postnatal days (PD) 4–9. Mice were sacrificed in adulthood (~PD 115), and surviving neurons in the olfactory bulb granular layer and brainstem facial nucleus were quantified stereologically.

Results: nNOS was expressed throughout postnatal development in olfactory bulb granule cells but was never expressed in the facial nucleus. In wild type mice, alcohol reduced neuronal survival to similar degrees in both cell populations. However, null mutation of nNOS more than doubled alcohol-induced cell death in the olfactory bulb granule cells, while the mutation had no effect on the facial nucleus neurons. As a result, in nNOS^{-/-} mice, alcohol caused substantially more cell loss in the olfactory bulb than in the facial nucleus.

Conclusions: Mutation of the nNOS gene substantially increases vulnerability to alcohol-induced cell loss in a brain region where the gene is expressed (olfactory bulb), but not in a separate brain region, where the gene is not expressed (facial nucleus). Thus, differences in

genotype may explain why some individuals are vulnerable to FASD, while others are not, and may determine the specific patterns of neuropathology in children with FASD.

INTRODUCTION

Maternal exposure to alcohol during pregnancy can lead to a variety of developmental problems in the offspring. These range from mild behavioral and learning deficits to severe physical and cognitive disabilities and are collectively known as Fetal Alcohol Spectrum Disorder (FASD) (Sokol et al., 2003; Hoyme et al., 2016).

Autopsy material (Jarmasz et al, 2017) and brain imaging studies (Nguyen et al., 2017) have revealed a multitude of diverse structural brain changes that result from prenatal alcohol exposure. These structural changes include microencephaly, neuronal ectopia, cerebellar hypoplasia, agenesis of the corpus callosum, reductions in gray matter and white matter volumes, altered cortical connectivity, and many others (Fryer et al., 2006). These differences in structural abnormalities are often paralleled by differences in neurological and cognitive deficits, so that ataxia is the most prominent symptom in some affected children, while behavior problems, attention deficit disorder, epilepsy, or learning problems are most notable in others (Boronat et al. 2017; Glass et al., 2017; Infante et al., 2015).

This diversity in outcomes is likely due to several factors. Among these are differences in the dose, pattern, and gestational timing of alcohol ingestion, as well as differences in maternal health, nutrition, and co-exposures (Bonthius and West, 1990; Shor et al., 2010; Coles et al, 2015; Coathup et al., 2017).

Genetic differences among fetuses also likely play a major role in determining differences in outcome following prenatal alcohol exposure (Warren and Li, 2005; Eberhart and Parnell, 2016; Goldowitz et al., 2014). Indeed, human twin studies have shown a much higher concordance rate for the diagnosis of FASD for monozygotic twins than for dizygotic twins (Streissguth and Dehaene, 1993; Christoffel and Salafsky, 1975; Riikonen, 1994). This finding suggests that genetics play a substantial role in determining susceptibility and resistance to alcohol-induced brain damage in-utero.

Within the brain, multiple trophic factors and signaling pathways can protect neurons and glia against a variety of insults (Gibon and Barker, 2017). Some of these endogenous pro-survival factors can protect developing brain cells against the neurotoxic effects of alcohol (Bonthius et al., 2003; Boschen and Klintsova, 2017). Furthermore, the expression of these factors and their receptors is genetically determined (Zunino et al., 2016). Thus, it is likely that genetic differences among individual fetuses in the timing, magnitude, isoform, and spatial expression of these protective factors underlie some of the inter-individual differences in the pattern of neuropathology observed in people with FASD. However, to date, there has been no specific gene identified whose mutation renders one brain region more vulnerable to alcohol-induced injury, while leaving another brain region unaffected.

Neuronal nitric oxide synthase (nNOS) is one gene that can protect developing neurons against alcohol toxicity (Bonthius et al., 2008; Karacay et al., 2007). The protein product of the nNOS gene (the enzyme, nNOS) exerts its protective effect by catalyzing the production

of nitric oxide (NO), thus stimulating the neuroprotective NO-cGMP-PKG pathway (Bonthius et al., 2008). However, the nNOS gene is not expressed in all neuronal populations (Yuan et al., 2006; Jinno and Kosaka, 2002; Gotti et al., 2005). Thus, it is possible that neuronal populations lacking nNOS will be particularly vulnerable to alcohol-induced cell death. One goal of this study was to test the hypothesis that a neuronal population not expressing nNOS will have more alcohol-induced cell death than a neuronal population that does express the protein.

Numerous studies have shown that mouse neurons carrying a null mutation of the nNOS gene (nNOS^{−/−}) have an increased vulnerability to alcohol-induced neuronal death (Bonthius et al., 2015; Karacay et al., 2015). This reflects the fact that those neuronal populations rely upon nNOS for neuroprotection. The null mutation of nNOS deprives those neurons of the protective mechanism that they would employ to promote their survival. However, because some neuronal populations do not normally express nNOS, null mutation of nNOS should not deprive the non-expressing neurons of neuroprotection and should not affect their survival. As a result, nNOS null mutations may greatly increase the vulnerability of some brain regions to alcohol toxicity, while not affecting others, thus potentially providing an example of genotype-dependent patterns of alcohol neuropathology. The second goal of this study was to test the hypothesis that null mutation of the nNOS gene will substantially increase the vulnerability of neurons in which nNOS is normally expressed, while having no effect on neurons in which nNOS is not normally expressed.

MATERIALS AND METHODS

Identification of nNOS-positive and nNOS-negative neuronal populations

Animals—Twenty-four wild type (129SVJ × C57/BL6) mouse pups, obtained from six separate litters, were used to determine the temporal and spatial expression of nNOS across the brain and to identify brain regions in which nNOS is either continuously expressed or continuously absent during postnatal development. The mouse pups were randomly assigned to groups sacrificed on postnatal day 1, 4, 7, 10, 14, 21, or 60 (n=3–4 per group). Males and females were included in each group. The Institutional Animal Care and Use Committee at the University of Iowa approved all of the procedures for the experiments described in this study.

Tissue preparation—On the day of sacrifice, the animals were anesthetized with ketamine, and perfused via the left cardiac ventricle with saline, followed by a cold fixative solution consisting of 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were extracted from the skull and stored in additional fixative at 4° C for at least three days. The forebrain, cerebellum, brainstem, and olfactory bulbs were isolated and placed in a cryoprotective 30% sucrose solution until they sank. Sections were then cut at 40 μm thickness using a freezing microtome. The forebrains and olfactory bulbs were cut in the coronal plane, the cerebellum in the parasagittal plane, and the brainstems in the orthogonal plane. The sections were saved in consecutive order in 96-well trays in a cryostorage solution until they were later processed for immunohistochemistry or histology.

Neuronal nitric oxide synthase (nNOS) immunohistochemistry—Immunostaining for nNOS protein was performed on floating sections. The sections were first incubated with 3% hydrogen peroxide for 30 min, followed by goat serum blocking solution for 60 min. The sections were then incubated with goat polyclonal anti-nNOS antibody (Abcam, Cambridge, UK, cat. number ab1376) (1:2,000) overnight at 4°C. Detection was performed with a biotinylated rabbit anti-goat IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pa.) followed by an avidin-biotin peroxidase complex for one hour and diaminobenzidine substrate (DAB substrate kit, Vector Laboratories SK-4100) for 5 minutes. Phosphate-buffered saline (PBS) was used for all dilutions and rinses. Negative controls for nNOS immunohistochemistry included omission of the primary nNOS antibody and use of brain sections from mice carrying an nNOS null mutation (nNOS^{-/-} mice described below).

For each section immunostained for nNOS, a nearby section was Nissl-stained. Examination of these Nissl-stained corresponding sections facilitated identification of the specific cell populations that were nNOS-positive and nNOS-negative in the immunostained sections.

Quantitation of nNOS immunostaining: Visual examination of the immunostained sections suggested that nNOS is expressed at all developmental time points in the olfactory bulb granule cell layer, while it is absent at all time points in the facial nerve nucleus. To verify this impression and to determine whether expression levels change across development in these specific regions, the intensity of nNOS immunostaining was measured for three animals (n=3) at each time point (PD 1, 4, 7, 10, 14, 21, and 60). One section from the brain stem and olfactory bulb of each animal were photographed digitally with a 10x objective. Utilizing the Image J image analysis system, the mean gray density was measured within a square 175 $\mu\text{m} \times 175 \mu\text{m}$, placed randomly at three target sites within the olfactory bulb granule cell layer and at three sites within the facial nucleus. To determine the background, the mean gray density was similarly measured at three sites on each olfactory bulb or brain stem, outside of the granule cell layer and facial nucleus, at which no cells were specifically stained. The value of the background staining was then subtracted from the mean gray value of each target site. The three values for each site were averaged together to yield one mean gray value for the olfactory bulb and one mean gray value for the facial nucleus for each pup.

Effect of nNOS null mutation on regional vulnerability to alcohol-induced neuronal loss.

Animals—This portion of the study utilized a strain of mice that is homozygous for a null mutation within the gene for neuronal nitric oxide synthase (nNOS^{-/-} mice). This strain was originally generated by homologous recombination (Huang et al., 1993). In a previous study, we used RT-PCR to verify that these mice do not express nNOS in any brain region (Bonthius et al., 2002). The nNOS^{-/-} mice have moderately abnormal gastrointestinal structure and function, but are fully viable, survive to adulthood, and reproduce. The gross brain morphology of these mutant mice is normal. Furthermore, we have shown that this strain of mice generates and maintains normal numbers of neurons within the hippocampus and cerebral cortex (Bonthius et al., 2004b; 2006) but has a modestly reduced number of cerebellar neurons, compared to controls (Bonthius et al., 2015).

129SVJ and C57B6 mice were the background strains of mice upon which the nNOS^{-/-} strain was generated. Therefore, for the wild type control, we utilized the F2 offspring of 129SVJ × C57B6 matings. These animals are recognized as the appropriate controls for the nNOS^{-/-} line (Dawson et al., 1996; Huang et al., 1993). Breeding pairs of nNOS^{-/-} and wild type mice (F1 offspring of 129SVJ × C57B6 matings) were obtained from Jackson Labs (Bar Harbor, Maine). All mice were bred and housed at the University of Iowa Animal Care Facility, which maintained a 12-hour lights on/lights off daily cycle.

Treatment Groups—A total of 47 mouse pups (24 wild type and 23 nNOS^{-/-}), derived from 6 wild type litters and 8 nNOS^{-/-} litters, were used for the cell count portion of this study. The pups were randomly assigned to one of three treatment groups, based on the daily dose of alcohol. Each treatment group for each genotype consisted of 7–8 subjects. Only male pups were used in this portion of the study. To minimize litter effects, a maximum of two pups per litter were assigned to any particular group.

All three treatment groups received intraperitoneal (ip) alcohol injections daily over postnatal days (PD) 4–9 (Bonhies et al., 2002). This timing of alcohol exposure in neonatal mice mimics human fetal brain exposure during the third trimester of gestation and corresponds to the “brain growth spurt,” a period during which the developing brain is particularly vulnerable to teratogens (Dobbing and Sands, 1979; Goodlett and Johnson, 1999). At 09:00 each day, the pups were weighed and given a single ip injection of an alcohol-containing solution in sterile phosphate buffered saline, warmed to 37° C (Bonhies et al., 2002). This daily acute alcohol exposure models the “binge” pattern of consumption commonly practiced by women who drink alcohol during pregnancy (Kesmodel and Kesmodel, 2002; Muckle et al., 2011). The IP route of administration was used because it allows for precise control of the alcohol dose and for rapid administration of alcohol with no need for sedation and minimal stress to the animals (Bonhies et al., 2002).

The daily alcohol doses administered were 0.0 (injected control), 2.2, or 4.4 mg ethanol per gram of body weight. These doses were administered as 0%, 10%, and 20% (v/v) solutions, respectively. The 2.2 and 4.4 mg/g doses were chosen because they produce moderate and high blood alcohol concentrations, respectively (Bonhies et al., 2002; 2006), and are in the range that are commonly observed among human alcohol abusers (Pelissier et al., 2014). Immediately following the daily alcohol injection, the pups were returned to their mothers and allowed to nurse. Between PD10 and PD21, the pups were left undisturbed with their mothers. The pups were weaned on PD21 and housed 2–3 per cage until adulthood.

Tissue preparation and cutting of sections—After reaching adulthood (PD 110–122), the mice were weighed, anesthetized with ketamine/xylazine, and perfused with paraformaldehyde fixative, as described above. The brains were removed and stored in fixative at 4°C for at least 24 hours.

The right olfactory bulb and the brain stem were isolated from the rest of the brain to allow stereological cell counts of olfactory bulb granule neurons and neurons of the facial nerve nucleus. These particular neuronal populations were chosen for analysis and comparison because of the immunohistochemistry results (shown below) demonstrating that, in wild

type animals, granule neurons of the olfactory bulb express nNOS throughout postnatal development, while neurons of the facial nucleus do not express nNOS at any postnatal age.

For cryoprotection, the right olfactory bulbs and brain stems were placed in a 30% sucrose solution in phosphate buffer and gently rocked continuously on a rocker table at room temperature for 48 hrs. The cryoprotected tissues were positioned on the stage of a freezing microtome and cut exhaustively at 40 μ m. The olfactory bulbs were cut in the parasagittal or coronal plane, while the brain stems were cut in the coronal plane. All of the serial sections generated were saved in consecutive order with one section per well in 96-well tissue culture trays containing 4% paraformaldehyde fixative. The tissue culture trays were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI) to prevent evaporation of the fixative, and the collected sections were stored at 4°C pending further processing.

Stereological Cell Counts—To quantify neurons of the olfactory bulb and facial nerve nucleus, we used the optical disector method of stereology. The technique of and rationale for the optical disector method have been described elsewhere (Gundersen, 1986; West et al., 1988; Bonthius et al., 2004b). The optical disector method requires determination of the volume containing the cells of interest, referred to as the “reference volume” (V_{ref}), and the density (N_v) of the cells within that volume. The total number of neurons (N) for any specific neuronal population is the product of the numerical density and reference volume.

$$N = N_v \times V_{ref} \quad (\text{equation 1})$$

To conduct the stereology, we used the frozen section protocol previously described in detail by our group (Bonthius et al., 2004b). The total number of olfactory bulb granule cells and neurons of the facial nerve nucleus were stereologically quantified in animals of all three treatment groups (0.0, 2.2, and 4.4 mg/g alcohol) and both genotypes (wild type and nNOS $-/-$). As shown in figure 1, the olfactory bulb granule cell layer is easily identifiable as the layer containing a multitude of densely-packed granule neurons immediately internal to the mitral cell and internal plexiform layers. The cells of the facial nerve nucleus are easily identified as the neurons with large perikarya arranged in an oval cluster in the ventral pons.

Throughout this study, systematic random sampling (Bonthius et al. 2004b; Gundersen and Jensen, 1987) was used at all levels of data collection. Table 1 shows the specific sampling parameters utilized for quantifying neurons of the olfactory bulb and facial nucleus.

Accurate determination of cell number by the optical disector method requires an accurate measurement of section thickness. The sections were originally cut at the thickness of 40 μ m. However, transition of the sections from the frozen state to the thawed state and from a cryoprotective solution to fixative provided the opportunity for section thickness to change after processing. As previously described (Bonthius et al., 2004b), we used an electronic microcator to verify that the sections were acceptably uniform and approximately 40 μ m thick. In this study, the mean measured thickness of the sections was 38.8 μ m (range from 34.0 to 43.0) for the olfactory bulb and 39.6 μ m (range from 35.0 to 44.0) for the brain stem.

Effect of nNOS null mutation on the pharmacokinetics of alcohol.

A third set of animals was utilized to determine peak and trough blood alcohol concentrations (BACs). Wild type and nNOS^{-/-} male mice (n=5–6 per treatment and genotype) were treated with alcohol with the same treatment paradigm as described above (0.0, 2.2, or 4.4 mg/g/day each day over PD 4–9). One hour following the alcohol administration on PD4, the tip of the tail was clipped, and a blood sample (20 µl) was collected. This sample represents the peak BAC on PD4 (Bonthius et al., 2002). A second sample from each animal was collected the following morning, just prior to the administration of the alcohol dose on PD5. This trough BAC assesses the extent to which the pups had cleared the alcohol dose administered the previous day. The procedure was repeated following administration of the alcohol dose on PD8. Thus, a total of 4 blood samples were collected from each mouse pup, representing the peak and trough BACs of PD4 and PD 8. Blood alcohol concentrations were determined enzymatically, as previously described (Bonthius et al., 2002).

Statistical analyses

Reference volumes, cell densities, and cell numbers were analyzed by multivariate ANOVA (MANOVA) using SPSS statistical software. Genotype and alcohol treatment were the between-subjects factors in these analyses. Alcohol-induced percent changes in cell number for each neuronal population and treatment group were calculated by subtracting the number of cells of each cell population (olfactory granule cells and facial nucleus neurons) from the mean cell number of that cell population in the untreated control group, then dividing the difference by the mean cell number of the untreated control group and multiplying by 100. These percent changes in cell number were analyzed by two-way ANOVA, with genotype and alcohol treatment as the grouping factors. Body weights and BACs were analyzed by repeated measures ANOVA. The within-subjects (repeated measures) factor was age (days) for the body weight analysis and time point (peak or trough for day 4 or 8) for the BAC analysis. For both the body weight and BAC analyses, genotype and alcohol treatment were the between-subjects factors. nNOS immunostaining intensities were analyzed by univariate ANOVA with postnatal age (1–60) and brain region (facial nucleus and olfactory bulb) as the fixed factors. For all analyses, the experimental unit was the individual pup. Post-hoc analyses were conducted with Tukey tests for multiple comparisons.

RESULTS

nNOS is expressed in olfactory bulb granule cells, but not in the facial nucleus.

Using immunohistochemistry, we first examined expression patterns of nNOS to identify a neuronal population that uniformly expressed nNOS and another population that uniformly did not express nNOS within the developing brain. We found that nNOS is expressed in all four of the major brain regions examined (forebrain, cerebellum, olfactory bulb, and brain stem). However, the pattern and intensity of staining differed among brain regions and among cell populations (Figure 2). In the forebrain, nNOS-positive cells were diffusely distributed throughout the cerebral cortex and basal ganglia. Labeled neurons tended to be intensely stained, creating a Golgi-like staining pattern that labeled cell bodies and neurites. In the cerebellum and olfactory bulbs, the granule cell layers were heavily and uniformly

labeled. In both the cerebellum and olfactory bulb, it appeared that virtually all granule cells of those regions were nNOS-positive. Because the granule cells of those regions have a high packing density, the morphology of the individual cells was difficult to discern. In contrast to the small granule cells, virtually all of which were nNOS-positive, the large projecting neurons (mitral cells of the olfactory bulb and Purkinje cells of the cerebellum) were mostly nNOS-negative. In the brain stem, large numbers of neurons were intensely stained. Many of these nNOS-positive neurons were present in clusters, corresponding to brain stem nuclei. Others were dispersed across the brain stem reticular formation.

These patterns of staining in the forebrain, cerebellum, olfactory bulb, and brain stem were observed across postnatal development. Developmental changes in the intensity or distribution of nNOS-positive cells did not appear to change substantially among the time points examined. Negative controls for nNOS immunohistochemistry, which included omission of the primary nNOS antibody and use of brain sections from mice carrying an nNOS null mutation (nNOS^{-/-} mice) had no specific staining (Figure 2), thus demonstrating that the primary antibody is specific for nNOS and that nNOS^{-/-} mice have a true null mutation.

The principal goal of this portion of the study was to identify a brain region in which a neuronal population was uniformly nNOS-positive and another in which the cells were uniformly nNOS-negative. In the olfactory bulb, the granule cell population was strongly and consistently nNOS-immunopositive at all time points. In contrast, the facial nucleus of the brain stem contained no nNOS-immunopositive cells at any time point.

To quantitatively verify that the olfactory bulb granule cells express nNOS, while the facial nucleus neurons do not, nNOS immunoreactivity was measured and compared in these two regions. As shown in Figure 3, nNOS immunoreactivity within the olfactory bulb granule cell layer far exceeded background levels at all time points across development. In contrast, nNOS immunoreactivity within the facial nucleus did not differ from background at any time point. ANOVA of these measurements revealed a significant effect of brain region [$F(1,42)=177.8$, $p<0.001$], but not a significant effect of postnatal age. Post-hoc analyses revealed that nNOS immunoreactivity was significantly greater in the olfactory bulb granule cell layer than in the facial nucleus at all time points ($p<0.05$). Thus, the olfactory bulb granule cells and the facial nerve nucleus neurons were the populations chosen for comparison of nNOS-positive and nNOS-negative neuronal populations, respectively, in the cell count studies.

BACs were similar in nNOS^{-/-} and wild type mice.

To determine whether null mutation of the nNOS gene affected alcohol pharmacokinetics, we measured peak and trough blood alcohol concentrations on PD4 and PD8. Acute administration of alcohol at a dose of either 2.2 or 4.4 mg per gram of body weight resulted in high peak blood alcohol concentrations (BACs), followed by near total clearance the following morning (Figure 4). This pattern of high peaks and low troughs was observed following alcohol dosing both on PD4 and PD8. The differences in BAC between peak and trough sample times were reflected by a significant effect of time in the repeated measures ANOVA [$F(3,16)=2184$, $p<0.001$]. However, the peak and trough BACs on PD8 were not

significantly different from those on PD4, indicating that the significant effect of time was due solely to the differences between peaks and troughs and not to differences between PD4 and PD8.

As expected, the larger dose of alcohol (4.4 mg/g) resulted in higher peak BACs than did the smaller dose (2.2 mg/g). These dose-related differences in peak BAC were verified by a significant effect of treatment [$F(1,18)=960$, $p<0.001$]. The 4.4 mg/g dose produced peak BACs in excess of 300 mg/dl, while the 2.2 mg/g dose produced peak BACs just under 200 mg/dl. Because the peak BACs were higher for the 4.4 mg/g dose than for the 2.2 mg/g dose, while the trough BACs were uniformly low for both doses, there was a significant treatment x time interaction [$F(3,16)=295$; $p<0.001$].

Most importantly for this study, null mutation of the nNOS gene did not affect alcohol pharmacokinetics. At all timepoints and at both alcohol doses, nNOS^{-/-} and wild type mice had similar BACs. As a result, there were no significant main or interactive effects of genotype in the repeated measures analysis of BACs.

Alcohol temporarily suppressed body growth in nNOS^{-/-} and wild type mice.

Following administration of alcohol to the mouse pups, they became intoxicated and were temporarily unable to nurse. Pups receiving the low (2.2 mg/g) and high (4.4 mg/g) doses typically did not nurse for one and two hours, respectively, following the alcohol administration. These time periods were similar for both genotypes. Despite the pups' intoxication, dams appeared to care for the pups normally in both genotypes.

To determine whether alcohol or null mutation of nNOS affects body growth, we measured the weight of the mice over PD 4–10 and at adulthood. Mice of all three treatment groups (0.0, 2.2, and 4.4 mg/g/day) and both genotypes (WT and nNOS^{-/-}) grew progressively from PD4 to adulthood (Table 2). The daily growth of the mice was reflected by a significant effect of age [$F(7,34)=379$; $p<0.001$]. However, the mice did not all grow at identical rates or to the same degree, as their body weights were affected by genotype and by alcohol treatment.

Over PD4–10, the nNOS^{-/-} mice were similar in body weight to the wild type mice. However, by adulthood, the nNOS^{-/-} mice were modestly, but consistently, smaller than wild type. In particular, the nNOS^{-/-} mice were approximately 6% smaller than wild type. This divergence in body weight between the genotypes from infancy to adulthood led to a significant age x genotype interaction [$F(7,34)=2.351$; $p<0.05$]. However, post-hoc analyses did not reveal any significant body weight differences among groups in adulthood.

Alcohol restricted body growth for both wild type and nNOS^{-/-} mice, leading to a significant effect of treatment group [$F(2,40)=3.619$; $p<0.05$]. However, the effects of alcohol on body growth were modest. Mean body weights increased each day, despite treatment with either alcohol dose. The effects of alcohol on body growth were also only temporary and occurred only on PD 9 and PD10. On PD 10, the high dose of alcohol restricted body growth by 18% in the wild type mice and by 21% in the nNOS^{-/-} mice. By adulthood, alcohol-treated mice had body weights similar to those of the no-alcohol groups.

This effect of alcohol on selective days led to a significant age x treatment interaction [$F(14,68)=3.915$; $p<0.001$].

In summary, both the nNOS mutation and alcohol treatment affected body growth. However, both of these effects were modest. The nNOS mutation affected body size only in adulthood, while alcohol affected body size only in the latter stages of infancy.

Mutation of nNOS worsened alcohol-induced neuronal losses in the olfactory bulb, but not in the facial nucleus.

To determine whether null mutation of nNOS alters the spatial patterns of alcohol-induced cell loss, we conducted stereological cell counts of an nNOS-positive and an nNOS-negative brain region. Because nitric oxide plays important roles in development and physiology, the absence of nNOS could directly alter the number of neurons within specific cell populations. However, as shown in Figure 5, in the absence of alcohol, nNOS $^{-/-}$ mice had equivalent numbers of olfactory granule cells and facial nucleus neurons as wild type mice. Therefore, the null mutation of nNOS alone did not cause neuronal deficits in these regions.

In contrast, alcohol did lead to reductions in cell numbers (Figure 5). This was demonstrated by a significant effect of treatment group in the MANOVA of cell numbers [$F(4,80)=9.2$; $p<0.001$]. For both the granule cell population [$F(2,41)=19.6$; $p<0.001$] and the facial nucleus neuron population [$F(2,41)=5.27$; $p<0.01$] alcohol treatment caused dose-related reductions in cell number. In all cases, the mice treated with the highest dose of alcohol had the lowest number of neurons. Thus, alcohol reduced cell numbers in both brain regions.

However, the *magnitude* of alcohol's effect was not equivalent for all groups. This was verified in the MANOVA by a significant genotype x treatment interaction [$F(4,80)=3.59$; $p<0.01$] and underlines the importance of genotype in determining alcohol's effects on these cell populations.

The extent to which alcohol affected the cell populations and genotypes differently can best be appreciated by examining alcohol-induced percent changes in cell number. As shown in Figure 6, alcohol caused much greater cell losses in the olfactory bulb than in the facial nucleus, and the effect depended on genotype.

In the facial nucleus, alcohol led to modest cell losses for both genotypes and at both doses of alcohol. Alcohol-induced cell losses were all less than ten percent. Facial nucleus cell losses were not significantly greater in the high dose group than in the low dose group, nor were they significantly greater in the nNOS $^{-/-}$ mice than in wild type.

A very different pattern of cell losses arose in the olfactory bulb. In the olfactory bulb, cell losses were strongly dose-dependent. For both genotypes, cell losses were significantly greater in the high-dose alcohol group than in the low dose group ($p<0.05$). Furthermore, in the olfactory bulb, cell losses depended on genotype. For both the low dose (2.2 mg/g) and high dose (4.4 mg/g) of alcohol, cell losses were significantly greater in the nNOS $^{-/-}$ mice than in wild type ($p<0.05$). In the wild type mice, the low dose of alcohol led to a 7% *increase* in cell number, while this same dose in the nNOS $^{-/-}$ mice led a 9% *decrease*. A further effect starkly illustrating the importance of genotype and brain region occurred at the

high alcohol dose. For mice receiving the 4.4 mg/g alcohol dose, olfactory cell losses were only 7.0% for the wild type mice but rose significantly to 18.9% for the nNOS^{-/-} mice. In contrast, the facial nucleus did not show this dose-dependent vulnerability. In the same animals in which olfactory losses were 18.9%, facial nucleus losses were significantly less at only 8.6% ($p<0.05$). These findings that the impact of nNOS mutation on alcohol-induced cell losses depended on brain region was reflected by a significant cell type x genotype interaction in the repeated measures analysis of alcohol-induced cell losses [$F(1,27)=25.4$; $p<0.001$].

The alcohol-related changes in cell number in this stereological study were due to alcohol-induced reductions in both cell densities and in reference volumes (Figure 5). Furthermore, as was true of the changes in cell number, the changes in cell densities and reference volumes were affected by genotype. These relationships were verified by significant effects of genotype ($p<0.001$), treatment ($p<0.001$) and a genotype x treatment interaction ($p<0.05$) in the analysis of cell densities, as well as by significant effects of genotype ($p<0.05$) and treatment ($p<0.01$) in the analysis of reference volumes.

DISCUSSION

This study revealed four important new findings. First, neurons of the facial nucleus do not express nNOS, yet they are not more vulnerable to alcohol than olfactory bulb granule cells, which do express nNOS. This finding demonstrates that, while nNOS expression is protective against alcohol toxicity, cell populations are not necessarily more vulnerable to alcohol in its absence. Second, the mutation of a single gene can substantially impact the regional pattern of neuropathology induced by alcohol within the developing brain. This finding may help to explain why certain brain regions are highly affected in some cases of human FAS while different brain regions are affected in others. Third, null mutation of the nNOS gene does not increase the vulnerability of all neuronal populations to alcohol-induced cell death. In particular, neurons of the facial nerve nucleus are not more susceptible to alcohol toxicity in nNOS^{-/-} mice than in wild type. This is the first example of a brain region whose vulnerability to alcohol during development is *not* enhanced by the deletion of nNOS. Fourth, null mutation of the nNOS gene greatly increases the vulnerability of olfactory bulb granule cells to alcohol-induced cell death. Following high-dose alcohol exposure, olfactory bulb granule cells have three times greater cell losses in mice deficient for nNOS than in wild type mice. This finding expands the list of neuronal populations whose vulnerability to alcohol is enhanced by genetic deficiency of nNOS (Karacay et al., 2015; Bonthius et al., 2015; de Licon et al., 2009).

One principal hypothesis examined in this study was that, in wild type mice, alcohol would cause greater cell losses in cell populations that do not express nNOS than in cell populations that do express nNOS. The facial nucleus was designated as the nNOS-negative population, while the olfactory bulb granule cells were designated as the nNOS-positive population. Thus, the hypothesis predicted that, in wild type mice, alcohol-induced cell losses would be greater in the facial nucleus neurons than in the olfactory bulb granule cells. However, this was not the case. For the wild type mice, cell losses in the facial nucleus were not significantly different from those in the olfactory bulb. Therefore, the results did not

support the hypothesis that absence of nNOS expression worsens neuronal vulnerability to alcohol *in wild type mice*.

The second principal hypothesis examined in this study was that null mutation of the nNOS gene would worsen alcohol-induced cell losses to a greater extent in cell populations that normally express nNOS than in those that don't. This hypothesis predicts that the nNOS null mutation will worsen alcohol-induced cell losses to a greater extent in olfactory bulb granule cells than in facial nerve nucleus neurons. The results strongly supported this hypothesis. In the olfactory bulb granule cell population, alcohol led to significantly greater percent cell losses in the nNOS^{-/-} mice than in the wild type mice for both alcohol doses. In contrast, in the facial nucleus, alcohol-induced cell losses were virtually equivalent in the two genotypes. Furthermore, in the nNOS^{-/-} mice that received the high alcohol dose, neuronal losses in the olfactory granule cell population were more than twice as severe as in the facial nucleus. Thus, mutation of the nNOS gene worsened the adverse effects of alcohol on the developing brain, but the effect was region-dependent. The effect was seen in olfactory bulb granule cells, where nNOS is normally expressed during development, but not in the facial nucleus, where nNOS is not expressed.

In summary, the results demonstrated that lack of nNOS does not increase vulnerability to alcohol in a neuronal population in which nNOS is not normally expressed (such as the facial nucleus neurons). However, null mutation of nNOS does increase vulnerability to alcohol in neuronal populations in which nNOS is normally expressed (such as the olfactory bulb granule cells).

Since the initial descriptions of fetal alcohol syndrome in the 1970s, it has been repeatedly observed that affected individuals vary substantially in the severity of brain injury induced by fetal alcohol exposure (Little and Streissguth, 1981, Mattson et al., 2011). This unequal vulnerability was graphically illustrated by a case report describing substantial discordance between a pair of fraternal twins, in which only one of the twins had fetal alcohol syndrome, while the other was spared (Christoffel and Salafsky, 1975). A subsequent study of 16 alcohol-exposed pairs of twin children found concordance for FAS in all five monozygotic twin pairs but discordance in 7 of the 11 dizygotic twin pairs (Streissguth and Dehaene, 1993). These findings suggested that genetics are a key determinant of alcohol teratogenesis (Warren and Li, 2005).

However, fetuses differ not only in the *severity* of pathology induced by alcohol but also in the *location* of the pathology. For example, neuroimaging studies have shown that, for some children with FASD, the cerebellar vermis is highly affected, while in other children with FASD, the vermis is spared and the corpus callosum, subcortical white matter, or some other brain region is the major target (Boronat et al., 2017; Donald et al., 2015). These regional differences in neuropathology among individuals are likely due, at least in part, to inter-individual differences in regionally expressed genes. A gene variant that renders cells more vulnerable to alcohol toxicity will lead to more alcohol-induced neuropathology in regions in which that gene is expressed than in regions in which it is not.

In this study, we found that null mutation of the nNOS gene worsens alcohol-induced cell losses from the olfactory bulb granule cell layer, where nNOS is normally expressed. In stark contrast, this same mutation has no effect on alcohol-induced cell loss in the facial nerve nucleus, where the gene is not expressed. Thus, this research provides proof-of-principle for the concept that genetic differences among individuals can explain regional differences in neuropathology among people with FASD.

In this study, nNOS was the specific gene whose mutation altered the regional patterns of alcohol-induced cell loss in the mouse model. Whether nNOS mutations similarly underlie differences among humans with FASD is unknown. A null nNOS mutation has not been described in humans. However, nNOS is a developmentally regulated gene, and its levels and sites of expression change substantially during gestation (Santacana et al., 1998, Ohyu and Takashima, 1998). In addition, the nNOS gene is differentially regulated in different neuronal populations and at different developmental stages. Furthermore, several polymorphisms of nNOS exist in humans, some of which increase susceptibility to a variety of diseases (Chung et al., 1996; Levecque et al., 2003; Serra et al., 2011). Thus it is possible that nNOS polymorphisms contribute to the variation in severity and regional pathology in human cases of FASD.

To date, nNOS is the only known gene whose mutation worsens alcohol toxicity in the developing brain. However, in light of the wide phenotypic variability of FASD, it is likely that many genes contribute to the protection against or vulnerability to alcohol-induced pathology. Indeed, several genes have been identified whose mutation can protect developing neurons against alcohol toxicity. These include BAX and tissue plasminogen activator (tPA), both of which are pro-apoptotic genes whose null mutation protects developing neurons against alcohol-induced cell death (Noel et al., 2011; Heaton et al., 2006). Other phenotypic aspects of FASD are likewise impacted by fetal genetics. In particular, alcohol-induced midface abnormalities can be worsened by mutations of platelet-derived growth factor receptor alpha (McCarthy et al., 2013) and several other genes (Swartze et al., 2014). Thus, for every alcohol-exposed fetus, there is likely a myriad of genes and genetic variations that individually and collectively add to and subtract from alcohol's teratogenic impact.

The mechanism by which nNOS exerts its protective effect against alcohol-induced cell death is unknown. However, several important mechanistic aspects have been discovered. nNOS catalyzes the production of nitric oxide (NO), which activates a particular signaling pathway involving a cyclic nucleotide and a protein kinase, the NO-cGMP-PKG pathway (Friebe et al, 1998). *In vitro* studies have shown that pharmacologic blockade of the NO-cGMP-PKG pathway at any step eliminates the protective effects of nNOS against alcohol, while activation of the pathway at any step promotes cellular survival (Bonthius et al., 2004a). To produce a neuroprotective effect, PKG phosphorylates a variety of effector molecules, whose identities are not all known. However, one known target of PKG is the transcription factor, NF-kappaB. The NO-cGMP-PKG pathway activates NF-kappa B to produce at least part of its neuroprotective effects against alcohol (Bonthius et al., 2008, 2009). It is likely that NF-kappaB regulates downstream neurotrophic and anti-apoptotic genes to produce the pathway's neuroprotective effects against alcohol.

This study has several limitations. First, the study was conducted only in male mice. Thus, whether similar region-dependent effects would be observed in females is unknown. However, in all of our previous studies examining the effect of nNOS gene deletion on alcohol vulnerability, males and females have been affected similarly (Bonthius et al., 2006; Klein de Licon et al., 2009; Bonthius et al., 2015; Karacay et al., 2015; Karacay and Bonthius, 2015). Second, the present study compared only one region in which nNOS is expressed (olfactory bulb) with one region in which nNOS is not expressed (facial nucleus). Whether all nNOS-expressing and non-expressing brain regions would show the same patterns as the olfactory bulb and facial nucleus is unknown. However, this limitation does not detract from the principal finding of the study, which is that mutation of a single gene can alter the regional patterns of vulnerability to fetal alcohol-induced damage. A third limitation of the present study is the fact that secondary changes may occur in the nNOS^{-/-} mice. Null mutation of nNOS can alter the expression of other genes and the function of signaling pathways, in both the central nervous system and elsewhere (Kim et al., 2004; Huang and Lo, 1998; Froehner et al., 2015). Thus, the different patterns of alcohol-induced pathology observed between the wild type and nNOS^{-/-} mice might not be due directly to NO or to nNOS.

The results of this study could have broad implications for understanding FAS neuropathology. Many trophic factors and protective signaling pathways exist within the central nervous system. However, most of these protective factors are expressed only in select brain regions or cell populations and not in others. Thus, variation in the expression of the genes encoding these protective factors would be expected to increase or decrease neuronal vulnerability to alcohol in a cell population-specific or region-specific manner and could explain the variation between children in pathology and clinical outcome of FASD.

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Olfactory Bulb

Brain Stem Facial Nucleus

Figure 1.

Olfactory bulb granule cells and facial nucleus neurons could be readily identified and quantified stereologically. Pictured here are Nissl-stained, 40 μ m thick, sections through the olfactory bulb (A, B) and brain stem (C, D) of an adult (PD 110) wild type mouse. A. The granule cell layer (region within the dotted line) of the main olfactory bulb is composed of small, densely packed and homogeneously dispersed cells. It is located interior to the external plexiform layer (EP) and mitral cell layer and is anterior to the accessory olfactory bulb (AOB) and olfactory tract (OT). B. Higher power view of the box in A shows that the granule cell layer (Gr) is sharply demarcated from the mitral cell layer (M) and internal plexiform layer (IP) that border it. The glomerular layer (Gl) and external plexiform layer (EP) are further external. C. The facial nuclei (F and arrows) are paired, oval-shaped structures at the ventral surface of the pons. They can be readily distinguished from the surrounding reticular formation (Ret). The inferior cerebellar peduncle (ICP) is lateral, while the medial vestibular nucleus (MV) is medial and dorsal. D. Higher power view of the box in C shows that the facial nucleus (F) is composed of large neuronal cell bodies that can be

readily distinguished from the small, diffuse cells of the adjacent reticular formation (Ret). Olfactory bulb granule cells and facial nucleus neurons were quantified stereologically utilizing the optical disector method of stereology. Magnification bars represent 1mm in A & C, and 200 μ m in B & D.

Figure 2.

Neuronal nitric oxide synthase (nNOS) is expressed in different patterns and levels of intensity in different brain regions. Wild type mouse brain sections (40 μ m thick) were Nissl-stained (A) or immunohistochemically stained for nNOS (B-H). The goal of this portion of the study was to find a brain region in which nNOS is consistently present in postnatal life and a brain region in which it is absent. These sections were taken from mice on PD 10, but immunostaining was similar at all ages over PD 1–60. A. Nissl-stained section through the brain stem demonstrating the location of the facial nerve nucleus (dotted line) in the ventral pons. Ret=reticular formation, MV=medial vestibular nucleus, ICP=inferior cerebellar peduncle. B. Nearby section immunostained for nNOS, demonstrating that the region of the ventral pons corresponding to the facial nucleus (box) is devoid of nNOS

staining. C. Higher-power view of the box in B demonstrating that cells of the facial nerve nucleus (F) are nNOS immune-negative, while some cells of the surrounding reticular formation (arrows) are nNOS immune-positive. D. Section through the olfactory bulb demonstrating that virtually all of the cells of the granule cell layer (Gr) are strongly nNOS-immunopositive. Gl=glomerular layer, E=external plexiform layer, M=mitral cell layer. In the cerebral cortex (E) and basal ganglia (F) nNOS immunostaining intensely labeled relatively widely-spaced cells in a Golgi-like fashion (arrows). G. In the dentate gyrus, nNOS immunostaining labeled cells diffusely within the granule cell layer and sporadically within the hilus (arrows). H. In the cerebellum, the granule cell layer (Gr) was strongly nNOS-immuno-positive, as were some of the cells within the molecular layer (M), while cells of the Purkinje cell layer (P) were nNOS-immuno-negative. Magnification bars represent 1 mm in A and B, 300 μ m in C, 200 μ m in D, 150 μ m in E and F, 100 μ m in G, 50 μ m in H, and 100 μ m in I-L.

Figure 3.

Neuronal nitric oxide synthase (nNOS) is expressed in olfactory bulb granule cells, but not in facial nucleus neurons. The intensity of nNOS immunostaining was measured in the facial nucleus of the brain stem and in the granule cell layer of the olfactory bulb of wild type mice of various ages. nNOS immunostaining was uniformly high across all ages in the olfactory bulb granule cell layer. In contrast, in the facial nucleus, nNOS immunostaining did not exceed background levels at any age.

*Significantly greater expression in the olfactory bulb than in the facial nucleus.

Figure 4.

Blood alcohol concentrations (BACs) were similar in nNOS^{-/-} and wild type mice. BACs were measured four times on each pup – at times corresponding to peak and trough BACs on postnatal days 4 and 8. Mouse pups receiving the higher alcohol dose (4.4 mg/g/day) had higher peak BACs than did those pups receiving the lower alcohol dose (2.2 mg/g/day). Trough BACs were uniformly low for both the high-dose and low-dose of alcohol. Wild type and nNOS^{-/-} mice had very similar BACs at all time points and for both alcohol doses. Thus, absence of the nNOS gene did affect alcohol pharmacokinetics.

Figure 5.

Alcohol reduced neuronal numbers in the olfactory granule cell layer, but not in the facial nucleus.

A. Stereological cell counts in the olfactory bulb granule cell layer and facial nucleus. In the absence of alcohol, neuronal numbers were similar for nNOS $-/-$ and wild type mice. Thus, absence of nNOS alone did not affect neuronal numbers in the olfactory bulb granule cell layer or in the facial nucleus. Alcohol reduced the number of neurons in the olfactory bulb

and in the facial nerve nucleus in mice of both genotypes. Thus, both cell populations were vulnerable to alcohol-induced cell death.

B. Cell densities, in the absence of alcohol, were similar for wild type and nNOS^{-/-} mice for both the olfactory granule cell layer and for the facial nucleus. Cell densities were significantly reduced by alcohol, but only in the olfactory bulb.

C. Reference volumes, in the absence of alcohol, were similar for wild type and nNOS^{-/-} mice for both the olfactory granule cell layer and for the facial nucleus. Reference volumes were significantly reduced by alcohol for both the olfactory bulb granule cell layer and the facial nerve nucleus and for both genotypes. Thus, the alcohol-induced cell losses were generally due to reductions in both the packing density of cells and the volume in which the cells are distributed.

* Significantly different from the 0.0 mg/g group ($p < 0.05$).

Significantly different from the 2.2 mg/g group ($p < 0.05$).

Figure 6.

Mutation of the nNOS gene worsened alcohol-induced neuronal losses in the olfactory bulb, but not in the facial nucleus. Shown here are percent changes in the number of neurons for the olfactory bulb granule cell layer and the facial nerve nucleus for wild type and nNOS^{-/-} mice following alcohol exposure. In the wild type mice, alcohol-induced changes in the olfactory bulb were similar to those in the facial nucleus. Thus, the facial nucleus was not more vulnerable to alcohol-induced cell losses despite its lack of nNOS expression. In the olfactory bulb, alcohol-induced percent cell losses were significantly more severe for nNOS^{-/-} mice than for wild type mice for both alcohol doses. In contrast, in the facial nucleus, alcohol-induced cell losses were equivalent for nNOS^{-/-} and wild type mice at both alcohol doses. Furthermore, for the nNOS^{-/-} mice, alcohol-induced percent cell losses were significantly greater in the olfactory bulb than in the facial nucleus. In contrast, in wild type mice, the olfactory bulb was not more vulnerable than the facial nucleus. Thus, null mutation of the nNOS gene enhanced the vulnerability of neurons to alcohol-induced cell loss, but the increased vulnerability was brain region-dependent.

* Significantly different from each other ($p < 0.05$).

Table 1.

Stereology sampling scheme used with the optical disector method to quantify neurons of the olfactory bulb granule cell layer and facial nerve nucleus.

	Olfactory bulb granule cells	Facial nerve nucleus
Section thickness	40um	40um
Disector height	12um	12um
Counting frame area	10um × 10um	50um × 50um
Raster pattern sampling step	1/500	1/2
x,y step	50000 um ²	5000 um ²
Section sampling fraction for Vref	1/4	1/3
Section sampling fraction for Nv	1/4	1/6

Table 2.

Body weights (grams) of wild type and nNOS^{-/-} mice treated with various doses of alcohol over PD 4–9.

Genotype	Alcohol Dose (mg/g/day)	Postnatal age (days)							
		4	5	6	7	8	9	10	Adult
Wild Type	0.0	2.67±0.08	3.29±0.09	3.83±0.08	4.53±0.09	5.00±0.08	5.61±0.14	6.09±0.16	32.45±1.13
	2.2	3.12±0.16	3.85±0.15	4.33±0.15	4.95±0.16	5.49±0.18	6.03±0.21	6.55±0.25	32.74±2.15
	4.4	2.88±0.09	3.10±0.10	3.48±0.14	3.91±0.13	4.24±0.14	4.60±0.17 *	4.97±0.18 *	33.36±1.75
nNOS ^{-/-}	0.0	3.17±0.16	3.85±0.14	4.42±0.16	5.13±0.19	5.83±0.23	6.51±0.27	6.93±0.32	30.73±1.21
	2.2	3.31±0.31	3.84±0.16	4.32±0.19	4.94±0.25	5.65±0.22	6.28±0.24	6.75±0.34	30.33±1.64
	4.4	3.00±0.21	3.38±0.28	3.66±0.28	4.12±0.31	4.62±0.39	5.02±0.36 *	5.45±0.38 *	30.98±1.86

Measures represent mean ± sem.

* = Significantly different from 0.0 mg/g/day group of the same genotype (p<0.05).