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Genetic absence of nNOS worsens fetal alcohol effects in mice.

I: Behavioral deficits

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Abstract

Background—Alcohol abuse during pregnancy often induces neuropsychological problems in the offspring, including learning disorders, attention deficits, and behavior problems, all of which are prominent components of fetal alcohol spectrum disorders (FASD). However, not all children who were exposed to alcohol in utero are equally affected by it. While some children have major deficits, others are spared. This unequal vulnerability is likely due largely to differences in fetal genetics. Some fetuses appear to have certain genotypes that make them much more prone to FASD. However, to date, no gene has been identified that worsens alcohol-induced brain dysfunction. Nitric oxide (NO) is a gaseous molecule that can protect developing neurons against alcohol-induced death. In the brain, NO is produced by neuronal nitric oxide synthase (nNOS). In this study, we examined whether homozygous mutation of the nNOS gene in mice worsens the behavioral deficits of developmental alcohol exposure.

Methods—Wild type and nNOS^{−/−} mice received alcohol (0.0, 2.2, or 4.4 mg/g) daily over postnatal days (PD) 4–9. Beginning on PD 85, the mice underwent a series of behavioral tests, including open field activity, the Morris water maze, and paired pulse inhibition.

Results—For the wild type mice, alcohol impaired performance only in the water maze. In contrast, for the nNOS^{−/−} mice, alcohol impaired performance on all three tasks. Furthermore, the nNOS^{−/−} mice were substantially more impaired than wild type mice in their performance on all three of the behavioral tests and at both the low (2.2) and high (4.4) doses of alcohol.

Conclusions—Targeted disruption of the nNOS gene worsens the behavioral impact of developmental alcohol exposure and allows alcohol-induced learning problems to emerge that are not seen in wild type. This is the first demonstration that a specific genotype can interact with alcohol to worsen functional brain deficits in an animal model of FASD.

Keywords

FASD; paired pulse inhibition; Morris water maze; open field activity; forebrain

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Introduction

Human fetuses differ markedly in their vulnerability to fetal alcohol spectrum disorders (FASD) (Streissguth et al., 1994). While some children exposed to alcohol in utero have microencephaly, mental retardation, attention deficits, and other functional brain disorders, others exposed to similar alcohol quantities are not affected (Abel, 1995). Why some fetuses are more vulnerable than others is not clear, but genetic differences likely contribute substantially. Indeed, human twin studies have suggested that fetal genetics play an important role in determining vulnerability to alcohol-induced teratogenesis (Streissguth and Dehaene, 1993; Christoffel and Salafsky, 1975).

The particular genes that confer resistance or vulnerability to alcohol-induced brain damage in human fetuses have not been identified. However, because alcohol exerts its neuroteratogenic effects by altering the development and survival of neurons, genes involved in neuronal development, function, and survival are the strongest candidates.

Mice have recently emerged as an excellent model system for the study of alcohol teratogenesis because their genomes can be studied and manipulated, and their brains are vulnerable to alcohol-induced damage. Indeed, several mouse strains have been identified that have greater or lesser vulnerabilities to alcohol-induced cell death (Chen et al., 2011; Heaton et al., 2006; Noel et al., 2011; Young et al., 2003). However, whether the increased vulnerabilities to cell loss in these strains translate into worsened *behavioral* deficits has not been explored.

Expression of the neuronal nitric oxide synthase (nNOS) gene is critically important for protecting developing neurons against alcohol toxicity (Karacay et al., 2007; Bonthius et al., 2008). Within neurons, nNOS catalyzes production of nitric oxide (NO), a gaseous molecule with myriad functions, including neurotransmission, regulation of vascular tone, and control of several intracellular signaling pathways (Charriaut-Marlangue et al., 2013). Mice genetically deficient for nNOS (nNOS^{-/-} mice) suffer greater acute neuronal losses than do their wild type counterparts following developmental alcohol exposure (Bonthius et al., 2006). The goal of the present study was to investigate whether absence of a competent nNOS gene worsens alcohol-induced learning, attention, and behavioral deficits.

Materials and Methods

Animals

The nNOS^{-/-} strain of mice was generated by homologous recombination (Huang et al., 1993). Utilizing RT-PCR, we have verified that the homozygous nNOS^{-/-} mice do not express nNOS in any brain region (Bonthius et al., 2002). These mice may exhibit some abnormal behaviors (Tanda et al., 2009; Nelson et al., 1995). Nevertheless, general behavior patterns and gross brain morphology are normal. Furthermore, these mutant mice generate and maintain normal numbers of hippocampal and cortical neurons (Bonthius et al., 2006).

The nNOS^{-/-} strain was generated on a background of 129SVJ and C57B6 mouse strains. Therefore, for the wild type control, we utilized the F2 offspring of 129SVJ × C57B6

matings. These animals are recognized as appropriate controls for the nNOS^{-/-} line (Dawson et al., 1996; Huang et al., 1993). Breeding pairs of nNOS^{-/-} and wild type mice were obtained from Jackson Labs. All mice for the study were bred and housed at the University of Iowa Animal Care Facility, where the Institutional Animal Care and Use Committee approved all of the procedures.

Treatment Groups

On postnatal day 4 (PD4), pups were randomly assigned to one of three treatment groups, based on the daily dose of alcohol. Each treatment group for each genotype/sex combination consisted of 7-10 subjects (Table 1). Males and females were included and constituted separate subgroups.

All three treatment groups received intraperitoneal (ip) injections daily over PD 4-9 (Bonthius et al., 2002). This timing 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). 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. This daily acute alcohol exposure models the “binge” pattern of consumption commonly practiced by women who drink alcohol during pregnancy (Kesmodel and Kesmodel, 2002).

The daily alcohol doses administered were 0.0, 2.2, or 4.4 mg ethanol per gram of body weight. The 2.2 and 4.4 mg/g doses produce mean peak blood alcohol concentrations of approximately 158 and 360 mg/dl, respectively (Bonthius et al., 2002; de Licon, 2009). Wild type and nNOS^{-/-} mice do not differ in their alcohol pharmacokinetics (Bonthius et al., 2002).

Behavior Studies

The pups were weaned on PD 21. They were housed 2-3 per cage until young adulthood (PD 85-90), when behavioral testing was begun. Females began testing without regard to estrus cycle position. The mice underwent a battery of behavior tests, beginning with open field activity, followed by the Morris water maze and prepulse inhibition. The investigators were masked to the genotype and treatment of each mouse.

Open Field Activity

Open field activity is a measure of locomotor behavior (Prut and Belzung, 2003), which detects hyperactivity, one of the hallmark behavioral disturbances in children with FASD (O'Malley and Nanson, 2002). Abnormal open field activity reflects disturbances in the function of forebrain structures, including basal ganglia, thalamus, hippocampus, and cerebral cortex (Vanderwolf et al., 1997).

The test was conducted according to protocol (Pierce and Kalivas, 2007). The apparatus consisted of a cubic test arena 57 cm on each side. The arena's floor was a plexiglass grid with lines spaced at 5 cm intervals in the x-y plane. Further details of the configuration are provided in the Supplementary Material.

On the first day, the test subjects were placed into the arena for 60 minutes to allow habituation. Activity measurements were not recorded on this day. The following day, the subjects were placed into the middle of the apparatus, and horizontal activity levels were measured by counting the line crossings, defined as events in which both front paws crossed a line in either the forward or backward direction. The number of line crossings was determined for three-minute intervals every 15 minutes beginning at time 0, 15, 30, and 45 minutes.

Morris water maze

The Morris water maze is a test of spatial memory abilities, which relies heavily on hippocampal function (Morris et al., 1982). The water maze was included in this study because learning and memory are prominently affected in children with FASD and because the hippocampus is often damaged following developmental alcohol exposure in humans and experimental animals (Norman et al., 2009; Bonthius et al., 2006).

The task was conducted according to an established protocol (Wenk, 2004). Details of the maze configuration are provided in the Supplementary Materials.

On the first day, mice were placed into the maze with a visible platform, and escape latencies were measured. This allowed verification that the mice of both strains and of all treatment groups could see and swim with roughly similar abilities and that they possessed roughly equal motivations for escape.

The following day, mice were placed into the maze with the invisible platform, and escape latencies were measured. If the mouse found the platform in less than 120 seconds, then it remained on the platform for 15 seconds. If the mouse failed to find the platform in less than 120 seconds, then it was guided to the platform and remained there for 15 seconds. Each mouse underwent four trials each day for seven consecutive days, with five-minute inter-trial intervals. For each trial, the time (in seconds) to find the submerged platform was measured.

The day following the seven-day acquisition phase, each mouse underwent a spatial probe trial, in which the mouse was placed into the maze with the platform removed. This tested the mouse's knowledge of the platform location. For the probe trial, the maze consisted of four quadrants: the "target" quadrant (which previously contained the escape platform), the "opposite" quadrant (180 degrees opposed to the target quadrant), and two "adjacent" quadrants (on either side of the target quadrant). Mice swam in search of the platform for 60 seconds, during which the time spent in the target quadrant and opposite quadrant were measured. The time spent in the adjacent quadrants was derived mathematically. The greater the proportion of time spent in the target quadrant, the greater the extent to which the mice had learned the location of the escape platform.

Prepulse Inhibition

Prepulse inhibition (PPI) is the normal reduction in startle magnitude that occurs when an abrupt startling stimulus is preceded by a weak prestimulus (prepulse). PPI reflects sensorimotor gating and depends upon proper forebrain function (Swerdlow et al., 2001). PPI

testing was included because sensorimotor dysfunction and forebrain maldevelopment are common in people with FASD and in animal models of the disorder (Norman et al., 2009; Astley et al., 2009; Xie et al., 2010).

PPI testing was conducted according to protocol (Geyer and Dulawa, 2003). Details of the PPI apparatus are provided in the Supplementary Materials.

Each animal underwent a single testing session, which began with a five-minute acclimation period, followed by 64 recorded trials. The stimuli presented in the trials included 1) a startling 120 dB auditory burst of white noise for 40 msec; 2) a 20 msec prepulse of 4, 8, or 16 dB of broad band noise presented 100 msec before the onset of the startling stimuli and 3) no stimuli. To prevent anticipation, the intertrial interval varied 8-23 seconds. All stimuli were presented in a pseudo-random order against a background of 65 dB. Percent prepulse inhibition (%PPI) for each intensity of prepulse stimuli (4, 8, and 16 dB) was calculated according to equation 1, where the pulse alone score is the average of the movement magnitudes for the pulse alone trials.

$$\%PPI = 100 \times [(pulse\ alone\ score) - (prepulse + pulse\ score)] / pulse\ alone\ score \quad \text{(Equation 1)}$$

Neuronal counts

Following the behavior studies, the brains were weighed, and neurons of the cerebral cortex and hippocampus were quantified stereologically. These neuropathological results are reported in the companion paper (Karacay et al., 2014).

Statistical analyses—All statistical analyses were conducted with SPSS software. For all analyses, genotype (wild type or nNOS^{-/-}), alcohol treatment (0.0, 2.2, or 4.4 mg/g), and sex were the between-subjects factors. Body weights over PD 4-10 were analyzed by linear mixed model analysis for repeated measures. Body weights at adulthood were analyzed by univariate ANOVA.

For the open field test, the number of line crossings for each of the four consecutive time periods was analyzed by linear mixed model analysis for repeated measures. The number of line crossings for the four three-minute intervals was also totaled and analyzed by univariate ANOVA.

Data from the training phase (days 1-7) of the water maze were analyzed by linear mixed model analysis for repeated measures. Data from the probe trials were analyzed by multivariate analysis of variance (MANOVA) with time in the target-, opposite-, and combined adjacent-quadrants as the dependent variables.

For the prepulse inhibition test, the startle intensities at baseline (in the absence of any prepulse) were analyzed by univariate ANOVA. Next, to determine how differences in prepulse intensity affected prepulse inhibition, the data were analyzed by repeated measures ANOVA with prepulse stimulus intensity (4, 8, and 16 dB) as the within-subjects (repeated measures) factor. To determine the effect of alcohol, genotype and sex on the prepulse

inhibition, the data were next analyzed by MANOVA with percent inhibition at each magnitude of the prepulse stimulus (4, 8, and 16 dB) as the dependent variables.

The effects of alcohol on the behavioral results were also expressed as a percent change from control. These alcohol-induced percent changes allowed visualization and analysis of the extent to which alcohol altered behavioral performance differently in the two mouse strains. The alcohol-induced percent changes for total line crossings in the open field test and prepulse inhibition were each analyzed by univariate ANOVA. For the probe trial in the water maze, the data were analyzed by MANOVA (with the target-, opposite-, and combined adjacent-quadrants as the dependent variables.) A significant main effect of genotype in these analyses would indicate that alcohol's effect on behavior depended on genotype.

All post-hoc analyses were conducted with Bonferroni adjustments for multiple comparisons.

Results

Body weight was affected by age, genotype, sex, and alcohol

Mice of both genotypes and of all treatment groups grew progressively from PD 4 to adulthood (Figure 1). The daily growth in body weight was confirmed by a significant effect of age [$F(6,72)=419.3$; $p<0.05$]. However, the mice did not all grow at the same rate, as their body weights were affected by genotype, sex, and alcohol. Over PD 4-10, mice of the two genotypes were similar in body weight. However, by adulthood, the wild type mice were larger than the *nNOS*^{-/-} mice. This led to a significant effect of genotype [$F(1,82)=7.573$; ($p<0.01$)]. Sex also affected body growth. Over PD 4-10, males and females of both genotypes had similar body weights. However, by adulthood, males were larger than females in both genotypes, thus leading to a significant effect of sex [$F(1,82)=34.6$; ($p<0.001$)].

Alcohol impaired body growth for both genotypes, leading to a significant effect of treatment group [$F(2,77)=8.97$; ($p<0.01$)]. However, the effects of alcohol on body growth were modest for wild type and *nNOS*^{-/-} mice, and the mean body weights increased each day, despite alcohol treatment. For both mouse strains, alcohol slowed body growth only in the animals administered the high dose (4.4 mg/g) and only after multiple days. Furthermore, alcohol's effect on body growth was temporary. By adulthood, body weights of the alcohol-exposed mice had caught up to those of the no-alcohol groups. This was confirmed by lack of any main or interactive effects of alcohol in the analysis of adult body weights.

Alcohol increased activity levels in *nNOS*^{-/-} mice, but not in wild type

Activity levels, as reflected by the open field test, were not affected by sex [(although others have found that females are more active than males (Chachua et al., 2014)]. However, none of the behavioral results in this set of studies showed any significant main or interactive effects of sex. Thus, the male and female data are combined in the figures showing the open field activity (Figure 2), water maze results (Figure 3) and prepulse inhibition (Figure 4).

Activity levels fell for mice of both genotypes and for all treatment groups with the passage of time (Figure 2). From period 1 through period 4, the number of line crossings in each successive period tended to decline for all groups. This was reflected by a significant effect of period [$F(3,80)=343.9$; $p<0.001$]. Thus, activity levels extinguished with time for both genotypes and all treatment groups.

In the absence of alcohol, wild type and nNOS $^{-/-}$ mice had similar patterns of activity. Among the mice administered no alcohol (0.0 mg/g), there were no significant differences between the wild type and nNOS $^{-/-}$ mice in line crossings in any of the four periods (Figure 2A and B) or in total line crossings (Figure 2C). Thus, at baseline (no previous alcohol), absence of the nNOS gene did not affect activity levels.

Exposure to alcohol increased activity levels, but the effect depended strongly on genotype. For the wild type mice, alcohol exposure did not significantly change activity levels. In contrast, for the nNOS $^{-/-}$ mice, alcohol exposure increased activity levels in the first, second, and fourth periods (Figure 2B) and increased total line crossings (Figure 2C) for mice administered the low (2.2 mg/g/day) and high (4.4 mg/g/day) doses. These differential effects of alcohol on the two genotypes led to significant genotype $\times$ treatment interactions in the repeated measures ANOVA of line crossings by period [$F(3,80)=5.387$; $p<0.005$] and in the univariate ANOVA of total line crossings [$F(2,82)=4.717$; $p<0.05$]. The different effect of alcohol on the two genotypes can best be appreciated in the plot of alcohol-induced changes in total line crossings (Figure 2D). Both the low and high alcohol doses increased activity levels to a much greater extent in the nNOS $^{-/-}$ mice than in wild type. This led to a significant effect of genotype [$F(1,56)=25.9$; $p<0.001$] in the ANOVA of alcohol-induced changes in activity levels. Thus, alcohol-induced hyperactivity, a key behavioral component of FASD, is worse in nNOS $^{-/-}$ mice than in wild type.

Absence of the nNOS gene impaired Morris maze performance. Alcohol worsened performance to a greater extent in nNOS $^{-/-}$ mice than in wild type

All of the treatment groups in both genotypes improved their water maze performances over time, as escape latencies declined over the seven sequential days of testing (Figure 3A). This improvement with experience was reflected by a significant effect of testing day [$F(6,77)=129.9$; $p<0.001$]. However, substantial differences in performance were evident among the groups. Even in the absence of alcohol, mice genetically deficient for nNOS did not perform as well as wild type in the acquisition phase of the water maze test. At baseline (no alcohol exposure), nNOS $^{-/-}$ mice were slower than wild type mice to find the platform on each of the seven testing days during the acquisition phase (Figure 3A). This difference between the genotypes was not likely due to differences in vision, swimming speed, or motivation, since escape latencies with the visible platform were similar (data not shown). Over the course of the acquisition phase, both the nNOS $^{-/-}$ and wild type groups learned the task, as their escape latencies declined. However, the nNOS $^{-/-}$ mice acquired the task more slowly than wild type (Figure 3A). By the time of the probe trial, the wild type and nNOS $^{-/-}$ mice had both mastered the task, as both groups spent substantially greater amounts of time searching in the target quadrant than in the opposite quadrant or either of the adjacent quadrants (Figure 3B). On the probe trial, performance of the nNOS $^{-/-}$ mice

did not differ from wild type. Thus, nNOS^{-/-} mice were impaired in their acquisition of spatial navigation, but could eventually learn the task.

Alcohol impaired performance on the water maze for the wild type and nNOS^{-/-} mice. During the acquisition phase (Figure 3A), previous exposure to alcohol increased escape latencies for both genotypes. Furthermore, on most trial days, the impairment was worse for the high dose than for the low dose alcohol treatment for both genotypes. This alcohol-induced impairment among both genotypes led to a significant effect of treatment [$F(2,82)=12.185$; $p<0.001$].

Alcohol impaired performance not only during the acquisition phase, but also during the probe trial (Figure 3B). For both genotypes, mice exposed to the high alcohol dose spent significantly less time searching in the target quadrant than did the mice exposed to no alcohol. Furthermore, for both genotypes, mice exposed to the high alcohol dose spent more time searching in the opposite quadrant than did the mice exposed to no alcohol. For the opposite quadrant, however, the differences were significant only for the nNOS^{-/-} mice. For both genotypes, alcohol also tended to increase the amount of time in the adjacent quadrants. These alcohol-induced impairments of performance were confirmed by a significant effect of treatment in the probe trial MANOVA [$F(4,162)=3.431$; $p<0.01$]. Tests of between-subjects effects confirmed significant effects of treatment group for the target ($p<0.002$), opposite ($p<0.005$) and adjacent quadrants ($p<0.05$).

While alcohol worsened performance for both genotypes, it had a greater negative impact on the nNOS^{-/-} mice than on wild type. During the acquisition phase (Figure 3A), the nNOS^{-/-} mice exposed to alcohol had greater escape latencies than any other group on all of the testing days. Most importantly, in the probe trial, alcohol worsened performance to a greater extent in the nNOS^{-/-} mice than in wild type (Figure 3C). In particular, at the low dose, alcohol led to a significantly greater reduction in target quadrant time and to a significantly greater increase in opposite quadrant time for the nNOS^{-/-} mice than for the wild type mice. Worsened performances by the alcohol-exposed nNOS^{-/-} mice were also seen at the high alcohol dose. This greater effect of alcohol on probe trial performance in the nNOS^{-/-} mice was confirmed in the MANOVA of alcohol-induced changes by a significant effect of genotype [$F(3,54)=20.58$; $p<0.001$]. Tests of between-subjects effects in this analysis confirmed significantly different alcohol effects for the two genotypes in the target quadrant ($p<0.005$), and in the opposite quadrant ($p<0.001$), but not in the adjacent quadrants. Thus, in a key test of learning and memory, alcohol-induced deficits were worse in nNOS^{-/-} mice than in wild type.

Alcohol impaired prepulse inhibition in nNOS^{-/-} mice, but not in wild type mice

At baseline (in the absence of a prepulse), all groups had similar startle intensities (data not shown). Thus, neither a previous exposure to alcohol nor absence of the nNOS gene directly affected the response to a startling stimulus. However, when the startle stimulus was preceded by a prepulse, group differences emerged (Figure 4). Delivery of a prestimulus (prepulse) led to substantial reductions (from as low as 15% to as high as 65%) in the magnitude of the startle response for animals of both genotypes and all treatment groups (Figure 4A). Furthermore, the magnitude of the inhibition depended upon the intensity of the

prepulse. While the 4 dB prepulse elicited only weak reductions in the startle response, the 16 dB prepulse elicited large reductions, and the intermediate 8 dB prepulse elicited intermediate reductions for both genotypes and all treatment groups. This dependence on prepulse intensity was confirmed by a significant effect of decibels [$F(2, 81)=468.9$; $p<0.001$]. Thus, prepulse inhibition of a startle response was elicited in both genotypes and in all treatment groups.

Alcohol exposure tended to reduce the magnitude of the prepulse inhibition. At 4 dB, where prepulse inhibition was minimal even in the absence of alcohol, exposure to alcohol had little or no effect. However, at the stronger prepulse stimuli (8 dB and 16 dB), alcohol tended to reduce the magnitude of prepulse inhibition in a dose-dependent fashion. This reduction in prepulse inhibition by alcohol was confirmed by a significant effect of alcohol treatment [$F(6,160)=2.370$; $p<0.05$].

Exposure to alcohol tended to reduce prepulse inhibition in both genotypes at the 8 dB and 16 dB levels. However, the effect was much stronger in the *nNOS*^{-/-} mice than in wild type. While alcohol tended to reduce prepulse inhibition in the wild type mice, the effects were statistically insignificant. In contrast, in the *nNOS*^{-/-} mice, alcohol significantly reduced prepulse inhibition at both the 8 dB and 16 dB levels of prepulse stimuli (Figure 4A).

The extent to which genotype impacted alcohol's effect on prepulse inhibition is most evident in the plot of alcohol-induced changes in prepulse inhibition (Figure 4B). Alcohol's effect on prepulse inhibition was stronger in the *nNOS*^{-/-} mice than in wild type. This was confirmed by a significant effect of genotype [$F(3,54)=5.451$; $p<0.005$]. At the 16 dB level of prepulse stimuli, the *nNOS*^{-/-} mice were significantly more impaired than wild type by the previous alcohol exposure. Thus, prepulse inhibition, a test reflecting forebrain function, was substantially more impaired by alcohol in mice genetically deficient for *nNOS* than in wild type mice.

Discussion

These studies showed for the first time that a single homozygous gene mutation can substantially worsen the functional deficits induced by alcohol exposure during development. The results underscore the importance of genetics and the potential impact of a single gene mutation in determining alcohol's neurobehavioral effects.

Immediately after the first description of fetal alcohol syndrome (Jones and Smith, 1973), it became apparent that some fetuses are far more prone than others to alcohol-induced brain injury. 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 FAS, while the other was spared (Christoffel and Salafsky, 1975). A subsequent study of 16 alcohol-exposed twins 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 play a strong role in determining vulnerability to alcohol teratogenesis (Warren and Li, 2005). The twin studies further suggested that *fetal* genotypes

are critically important in determining vulnerability to FAS, though other studies have illustrated a role for maternal genetics (Gilliam and Irtenkauf, 1990; Downing and Gilliam, 1999).

Animal studies exploring the role of genetics in FAS have confirmed genotype as an important factor. Strain-related differences in alcohol-induced structural birth defects and behavioral outcomes have been documented among strains of mice and rats (Chen et al., 2011; Downing et al., 2009; Gilliam et al., 1988; Ogawa et al., 2005; Riley et al., 1993; Thomas et al., 2000). However, these studies have left unclear the relative contributions of paternal, maternal, and fetal genotypes. Most importantly, most of these animal studies have been conducted on rodent strains that differ from each other in *multiple unidentified* genes. Thus, the specific genes responsible for the differences in vulnerability have remained unidentified.

Human studies examining the role of genetics in FAS have focused on the enzymes involved in alcohol metabolism. These studies have revealed that polymorphisms in alcohol and aldehyde dehydrogenases are correlated with the incidence of FAS (McCarver et al., 1997; Viljoen et al., 2001; Khaole et al., 2004; Stoler et al., 2002). However, it remains unclear whether the unequal rates of FAS associated with these isozymes are due to differences in alcohol metabolism or consumption, or unidentified genes in linkage disequilibrium with them (Warren and Li, 2005).

While genes encoding alcohol metabolizing enzymes play a role, they are not the only genes, and probably not even the most important ones, determining fetal risk of FASD. In light of the centrality of neuronal loss in the pathogenesis of FASD, it is likely that genes involved in the development, function, and survival of neurons play critical roles in determining alcohol's impact on the fetal brain.

For this reason, we explored the importance of the nNOS gene in determining vulnerability to alcohol teratogenesis. nNOS is expressed widely throughout the central nervous system, where it catalyzes production of nitric oxide (NO), a membrane-permeant gaseous molecule that plays a critical role in a wide variety of functions (Charriaut-Marlangue et al., 2013).

We found that targeted disruption of the nNOS gene alone had little effect on brain function. However, lack of nNOS substantially worsened alcohol's neuroteratogenic effects. Compared to wild type, mice that were genetically deficient for nNOS had worsened behavioral deficits following developmental alcohol exposure. These mutant mice also have worsened microencephaly and neuronal losses, as shown in the companion paper (Karacay et al., 2014). Some of these alcohol-induced differences in outcome between wild type and nNOS^{-/-} mice were not only *quantitative*, but *qualitative*. In particular, prepulse inhibition and open field activity were not affected by alcohol in the wild type mice, but were markedly abnormal in the nNOS^{-/-} mice. Thus, absence of nNOS gene function enhanced alcohol-induced deficits that were evident in wild type and produced additional effects that were not present in the wild type. This finding mimics the human condition, in which some children exposed in utero to alcohol have learning, attention, and behavior problems, while

other similarly exposed children are normal (Abel, 1995; Streissguth et al., 1994). These qualitative outcome differences are likely due, in large part, to genetic differences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Alcohol temporarily impaired body growth for both genotypes

Body weights of wild type mice (A) and nNOS^{-/-} mice (B) are plotted for each of the treatment groups. Over PD 4-10, male and female body weights did not differ from each other. Therefore, for those days, the male and female data are combined. By adulthood, in both genotypes, male body weights exceeded female body weights, so the sexes of the adults are plotted separately. Daily exposure to alcohol over PD 4-9 significantly reduced body growth for wild type and nNOS^{-/-} mice, but only in the groups receiving the high dose (4.4 mg/g/day) and only at the later time points. By adulthood, body weights of the alcohol-exposed mice had caught up to the unexposed mice. In adulthood, the nNOS^{-/-} mice were significantly smaller than wild type mice. All measures represent means. Error bars represent standard error of the mean.

* Significantly different from unexposed mice of the same genotype ($p < 0.05$).

Significantly different from wild type mice of the same sex ($p < 0.05$).

Open Field Activity

Figure 2. Alcohol induced hyperactivity in the nNOS^{-/-} mice, but not in wild type mice

Open field activity was measured in a cubic test arena, the floor of which consisted of a grid with lines spaced at 5 cm intervals in the x-y plane. Horizontal activity levels were measured by counting the number of line crossings for three-minute intervals every 15 minutes beginning at time 0, 15, 30, and 45 minutes. Line crossings for each of the time periods are plotted for wild type mice (A) and nNOS^{-/-} mice (B). In the absence of alcohol, line crossings for the two genotypes were similar. Alcohol significantly increased activity levels for the nNOS^{-/-} mice, but not for wild type mice, in three of the four time intervals. The

number of total line crossings (C) was significantly increased by both the low and high alcohol doses in the nNOS^{-/-} mice, but neither dose significantly increased total line crossings in wild type mice. Alcohol-induced percent changes in activity (D) were significantly greater in the nNOS^{-/-} mice than in wild type mice for both alcohol doses. A, B, and C: * significantly different from the no alcohol group of the same genotype ($p < 0.05$).

D: * significantly different from the wild type group that received the same alcohol treatment ($p < 0.05$).

Water maze

Figure 3. Developmental alcohol exposure worsened performance in the water maze to a greater extent in nNOS^{-/-} mice than in wild type mice

The Morris water maze consisted of a pool filled with an opaque liquid and an invisible escape platform submerged beneath the liquid's surface. A. Escape latencies were measured in four trials per day for seven consecutive days. Even in the absence of alcohol, nNOS^{-/-} mice did not perform as well as wild type in the acquisition phase of the water maze test, as the nNOS^{-/-} mice were slower than wild type mice to find the platform on each of the seven testing days during the acquisition phase. Alcohol substantially increased escape latencies for both the wild type and nNOS^{-/-} mice. B. For the probe trials, the escape platform was removed, and the percent of time spent in the target and non-target (opposite and adjacent) quadrants was measured. For both genotypes, mice exposed to the high alcohol dose spent significantly less time searching in the target quadrant than did mice exposed to no alcohol. Following the high alcohol dose, the nNOS^{-/-} mice, but not the wild type mice, spent significantly more time in the opposite (non-target) quadrant than did their non-exposed counterparts. C. Percent changes in probe trial performance due to alcohol showed that, for both the low and high alcohol doses, alcohol impaired performance to a greater extent in the nNOS^{-/-} mice than in wild type. At the low alcohol dose (2.2 mg/g/day), the nNOS^{-/-} mice spent significantly less time in the target quadrant and more time in the nontarget (opposite) quadrant than did the wild type mice. At the high alcohol dose (4.4 mg/g/day) the two genotypes did not differ significantly in time spent in the target quadrant,

but the nNOS^{-/-} mice did spend significantly more time than the wild type in the opposite quadrant. Thus, alcohol worsened performance for both genotypes, but had a greater negative impact on the nNOS^{-/-} mice than on wild type.

B: * significantly different from the no-alcohol group of the same genotype ($p < 0.05$).

C: * significantly different from wild type mice that received the same alcohol treatment ($p < 0.05$).

Figure 4. Alcohol exposure during brain development impaired prepulse inhibition in nNOS^{-/-} mice, but not in wild type mice

Prepulse inhibition is the normal reduction in startle magnitude that occurs when a sudden startling stimulus is preceded by a weak pre-stimulus (prepulse). Prepulses of 4, 8, or 16 dB of broad band noise were presented 100 msec before the onset of a startling acoustic stimulus, and the degree of motoric startle was measured with an accelerometer. Percent inhibition was calculated by comparing the startle responses in the presence and absence of the prepulse. A. The magnitude of inhibition depended on the strength of the prepulse (4 dB, 8 dB, or 16 dB) and on the previous dose of alcohol. As the strength of the prepulse

increased from 4 dB to 16 dB, the magnitude of the inhibition increased for both genotypes. As the dose of alcohol increased, the magnitude of the prepulse inhibition decreased. B. Alcohol-induced reductions in prepulse inhibition were substantially greater for nNOS^{-/-} mice than for wild type mice. This was especially true at the 16 dB prepulse, where nNOS^{-/-} mice were significantly more impaired than wild type by the previous alcohol exposure. Thus, prepulse inhibition was more impaired by alcohol in the nNOS^{-/-} mice than in wild type mice.

A: * significantly different from the no-alcohol group of the same genotype ($p < 0.05$). ** significantly different from the no-alcohol group of the same genotype ($p < 0.01$).

B: * significantly different from wild type mice that received the same alcohol treatment ($p < 0.05$).

Table 1

Treatment groups and number of subjects per group.

Genotype	Alcohol dose (mg/g/day)	Gender	n for the body weight and behavior studies
Wild Type	0.0 (injected control)	Male	8
		Female	7
	2.2	Male	8
		Female	9
	4.4	Male	10
		Female	7
nNOS ^{-/-}	0.0	Male	8
		Female	7
	2.2	Male	7
		Female	8
	4.4	Male	8
		Female	7