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Importance of genetics in fetal alcohol effects: null mutation of the nNOS gene worsens alcohol-induced cerebellar neuronal losses and behavioral deficits

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Abstract

The cerebellum is a major target of alcohol-induced damage in the developing brain. However, the cerebella of some children are much more seriously affected than others by prenatal alcohol exposure. As a consequence of *in utero* alcohol exposure, some children have substantial reductions in cerebellar volume and corresponding neurodevelopmental problems, including microencephaly, ataxia, and balance deficits, while other children who were exposed to similar alcohol quantities are spared. One factor that likely plays a key role in determining the impact of alcohol on the fetal cerebellum is genetics. However, no specific gene variant has yet been identified that worsens cerebellar function as a consequence of developmental alcohol exposure. Previous studies have revealed that mice carrying a homozygous mutation of the gene for neuronal nitric oxide synthase (nNOS^{-/-} mice) have more severe acute alcohol-induced neuronal losses from the cerebellum than wild type mice. Therefore, the goals of this study were to determine whether alcohol induces more severe cerebellum-based behavioral deficits in nNOS^{-/-} mice than in wild type mice and to determine whether these worsened behavior deficits are associated with worsened cerebellar neuronal losses. nNOS^{-/-} mice and their wild type controls received alcohol (0.0, 2.2, or 4.4 mg/g) daily over postnatal days 4–9. In adulthood, the mice underwent behavioral testing, followed by neuronal quantification. Alcohol caused dose-related deficits in rotarod and balance beam performance in both nNOS^{-/-} and wild type mice. However, the alcohol-induced behavioral deficits were substantially worse in the nNOS^{-/-} mice than in wild type. Likewise, alcohol exposure led to losses of Purkinje cells and cerebellar granule cells in mice of both genotypes, but the cell losses were more severe in the nNOS^{-/-} mice than in wild type.

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Behavioral performances were correlated with neuronal number in the nNOS^{-/-} mice, but not in wild type. Thus, homozygous mutation of the nNOS gene increases vulnerability to alcohol-induced cerebellar dysfunction and neuronal loss. nNOS is the first gene identified whose mutation worsens alcohol-induced cerebellar behavioral deficits.

Keywords

balance beam; rotarod; Purkinje cells; granule cells; cell death; microcephaly

Introduction

Shortly after fetal alcohol syndrome (FAS) was first described in 1973, it became apparent that not all children are equally affected by *in utero* alcohol exposure (Christoffel and Salafsky, 1975; Hanson et al., 1978). The components of the syndrome, which include prenatal and postnatal growth deficiencies, a characteristic set of midface abnormalities, and evidence of central nervous system dysfunction, are all readily evident in some alcohol-exposed children, but not in others (Abel, 1995). Furthermore, the central nervous system dysfunction differs markedly among children (Jacobson et al., 1998; Streissguth et al., 1994). Some children with FAS have major cognitive deficiencies, while others are far less affected (Jacobson et al., 1998). Still others have no learning disabilities, but have attention deficits, behavior problems, epilepsy, or ataxia as their major CNS manifestation of brain dysfunction. This realization that children prenatally exposed to alcohol are diverse in the nature and severity of their signs and symptoms led to the concept of fetal alcohol spectrum disorder (FASD) – an umbrella term that recognizes the wide range of outcomes following maternal alcohol use and abuse during pregnancy (Hoyme et al., 2005).

Many factors contribute to the diversity of outcomes in FASD. Among these are the dose and pattern of alcohol consumption, gestational timing of alcohol exposure, health and nutritional status of the mother, maternal abuse of other drugs besides alcohol, and the presence and timing of educational opportunities and interventions during postnatal life (Bonthius et al., 1988; Bonthius and West, 1990; Goodlett and Johnson, 1999; Jacobson et al., 2004; Thomas et al., 1998).

Another factor that likely plays a key role in the variability of prenatal alcohol effects is genetics (Warren and Li, 2005). However, despite its importance, the impact of genetics on FASD has been only poorly investigated. The few human studies examining the role of genetics in FASD have focused on maternal genes encoding enzymes involved in alcohol metabolism. These studies found that polymorphisms in the maternal genes for alcohol dehydrogenase and aldehyde dehydrogenase are correlated with the incidence of FASD (Viljoen et al., 2001; Stoler et al., 2002). Thus, maternal genetic differences likely play a role in determining fetal outcome of gestational alcohol exposure.

However, the most important genetic differences influencing vulnerability to FASD might not lie in the *maternal* genes, but in the *fetal* genes. Some fetuses likely express specific gene variants and gene combinations that make them substantially more vulnerable or less vulnerable than others to alcohol's teratogenic effects. Indeed, a clinical twin study has

demonstrated that the concordance for FASD is much greater in monozygotic twins than in dizygotic twins, thus strongly suggesting that fetal genetics is critically important in determining the outcome of prenatal alcohol exposure (Streissguth and Dehaene, 1993).

The specific genes that influence human vulnerability to alcohol teratogenesis are unknown. To uncover these genes, mouse models of FASD may be especially useful because the developing mouse brain is vulnerable to alcohol-induced damage and because the mouse genome can be readily studied and manipulated.

Using both genetic and pharmacological approaches, we have previously demonstrated that neuronal nitric oxide synthase (nNOS) is a gene whose function is critical in protecting the developing brain against alcohol-induced injury (Bonthius et al., 2004a, 2008; 2009). In particular, pharmacologic agents that activate nNOS, such as NMDA (N-methyl-D-aspartate) or that mimic nNOS action, such as DETA-NONOate, can protect neurons against alcohol-induced cell death *in vitro* (Bonthius et al. 2003; 2004). Likewise, genetic approaches that overexpress nNOS in cultured neurons can ameliorate alcohol-induced toxicity (Karacay et al., 2007). Conversely, agents that inhibit nNOS function, such as L-NAME (L-N^G-Nitroarginine methyl ester) worsen alcohol-induced cell death *in vitro*, and homozygous loss-of-function mutation of the nNOS gene (nNOS “knockout”) worsens alcohol-induced neuronal death *in vivo* (Bonthius et al., 2006; 2009; de Licona et al., 2009).

Among brain regions, the cerebellum is particularly vulnerable to alcohol-induced neuronal injury (Bookstein et al., 2006; Bonthius and West, 1990). Furthermore, the nNOS gene is expressed in the developing cerebellum, and homozygous null mutation of the nNOS gene worsens alcohol-induced cerebellar neuronal losses, both *in vitro* and *in vivo* (Bonthius et al., 2002). However, whether this “knockout” of the nNOS gene worsens the *functional* effects of alcohol in the cerebellum is unknown. Thus, the principal goal of this study was to determine whether homozygous mutation of the nNOS gene worsens alcohol-induced behavioral deficits mediated by the cerebellum.

We have previously shown that genetic deficiency of nNOS acutely worsens alcohol-induced cerebellar neuronal losses (Bonthius et al., 2002). However, nNOS, through its production of nitric oxide (NO), influences many neuronal functions besides neuronal survival, including neurotransmission, intracellular signaling pathways, neurogenesis, and synaptogenesis (Hu and Zhu, 2014). Thus, nNOS deficiency could worsen alcohol-induced behavioral deficits through a variety of mechanisms. Furthermore, in our previous study, the effects of nNOS deficiency on alcohol-induced cell numbers were determined in infant mice, immediately following the alcohol administration (Bonthius et al., 2002). Thus, the neuronal deficits observed acutely might not be permanent and might not underlie any behavioral deficits detected in adulthood. Therefore, the second major goal of this study was to determine whether genetic deficiency for nNOS permanently worsens alcohol-induced neuronal losses in the cerebellum and whether these neuronal losses are correlated with the behavioral deficits.

Materials and Methods

Animals

This study utilized a strain of mice that were 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 have utilized RT-PCR to verify that these mice do not express nNOS in the cerebellum or any other brain region (Bonthius et al., 2002). nNOS^{-/-} mice have moderately abnormal gastrointestinal structure and function. However, they are fully viable, survive to adulthood, and reproduce. The mutant mice may exhibit some abnormal behaviors (Tanda et al., 2009; Nelson et al., 1995; Weitzdoerfer et al., 2004). However, their general behavior patterns and gross brain morphology are 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).

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 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. 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. The Institutional Animal Care and Use Committee at the University of Iowa approved all of the procedures for this study.

Treatment Groups

A total of 91 mouse pups (46 wild type and 45 nNOS^{-/-}) were used for the behavioral and anatomical components of this study. On postnatal day 4 (PD4), litters were culled to a maximum of eight pups per litter, with four males and four females, when possible. The 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 7–8 subjects. Males and females were included and constituted separate subgroups, based on previous studies demonstrating sex differences in alcohol-induced behavioral and neuropathological changes (Sickmann et al, 2014; Varlinskaya and Mooney, 2014). The mouse pups were derived from seven wild type litters and eight nNOS^{-/-} litters. An effort was made to minimize litter effects. To accomplish this, a maximum of two pups per litter were assigned to any particular group or subgroup. Thus, each subgroup was derived from a minimum of five different litters, and each treatment group was derived from a minimum of six different litters.

All three treatment groups received intraperitoneal (ip) alcohol injections daily over postnatal days (PD) 4–9 (Bonthius 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 (Bonthius 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). The IP route of administration was used because it allows for precise control of the alcohol dose and for rapid and simple administration of the alcohol with no need for sedation (Bonthius 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, that are also pharmacologically relevant (Bonthius et al., 2002; 2006). Immediately following the daily alcohol injection, the pups were returned to their mothers and allowed to nurse.

Behavior Studies

After the last alcohol administration, the pups were left undisturbed with their mothers until they were weaned on PD 21. They were housed 2–3 per cage with mice of the same sex, but irrespective of treatment group, until young adulthood (PD 85–90), when behavioral testing was begun. For the female mice, the testing was conducted based on age, not on position in the estrus cycle. The mice underwent two tests of cerebellar function. These included the balance beam, followed by the rotarod. (The mice also underwent several tests of forebrain function, including the water maze, open field, and paired pulse inhibition, the results of which will be included in a separate report that focuses on the forebrain.) The behavioral tests were begun at 09:00 each day. The examiners conducting the behavioral tests and the cell count studies (described below) were blind to the treatment status of the mice.

Balance beam

The balance beam is a test of balance and coordination that relies critically, though not exclusively, on cerebellar function. In this test, mice must traverse an elevated narrow beam to reach an enclosed escape platform. Performance on this test is gauged by how quickly the mice can traverse the narrow beams of various widths and shapes. To conduct this test, we constructed a balance beam apparatus and utilized a standard published protocol (Carter et al., 2001). The apparatus consisted of one-meter beams of wooden dowels with a goal box on one end. The goal box, which was constructed of plywood and measured 20 cm per side, had a 4×5 cm entrance hole on one side. The start section of the beams and the goal box were supported by wooden stands, which maintained the apparatus at 50 cm above the surface of the ground. The interchangeable beams of wood had cross-sections that were either square (5, 12, and 25 mm wide) or round (11, 17, and 25 mm in diameter) and had lines drawn circumferentially around them 10 cm from either end. The entire apparatus was illuminated with a 60-W desk lamp located above and to one side of the start position of the beam. This bright light created an aversive stimulus, which the mice could escape by traversing the beam and entering the goal box.

To conduct the test, the mice were placed at the start end of the beam, facing the goal box, and the time required to traverse the beam was measured manually by an examiner. Each

mouse underwent three days of training, followed by a single day of testing, as described in a published protocol (Carter et al., 2001). For the training phase, only the 12-mm-wide square beam was used. Each mouse was placed at the start end of the beam and was given up to 60 sec to traverse the beam. If the mouse stopped while traversing the beam during the training phase, it was given encouragement with the nudge of a finger. Each mouse was given four consecutive trials on each of the three training days.

On the fourth day, the mice underwent test trials. Each mouse underwent two consecutive trials on each of the square and round beams. The mice were first tested on the beams of square cross-section, progressing from widest to narrowest. The mice were next tested on the round beams, again progressing from widest to narrowest. The time required to traverse the 80-cm central portion of the beam was measured manually for each test trial.

Rotarod

The rotarod test, which examines an animal's ability to balance on a rotating rod, is widely used to assess motor coordination and depends heavily on cerebellar function. These experiments utilized a commercially available rotarod apparatus (Ugo Basile Biological Research Apparatus, Varese, Italy), and the training and testing was conducted according to a published protocol (Carter et al., 2001). The 30-mm diameter rotating cylinder was equipped with spacer disks that allowed up to 6 mice to be tested simultaneously. The apparatus was also equipped with a fall sensor and a built-in latency-to-fall timer for each lane. The mice underwent three consecutive days of training, during which they were given four trials per day, with a 5–10 minute inter-trial interval. For each trial, the mice were placed facing forward on the rotating cylinder, and the cylinder was accelerated from 4 to 40 rpm over four minutes. The mice were left on the apparatus for a maximum of 240 seconds.

Following the training period, the mice underwent testing, consisting of three trials per day over four consecutive days. Again, the cylinder was accelerated from 4 to 40 rpm over four minutes. The time at which the mice fell from the rotating rod was automatically measured and displayed by the apparatus, and was recorded by the examiner. If the mice reached the four minute mark without falling, then they were removed from the apparatus and assigned a time of 240 seconds.

Tissue preparation and cutting of sections—Following completion of the behavioral testing, the adult mice were weighed, anesthetized with pentobarbital, and perfused via the left cardiac ventricle with 0.9% NaCl, followed by 4% (w/v) paraformaldehyde fixative in 0.1 M phosphate buffer (pH=7.4). The brains were removed and stored in fixative at 4°C for at least 24 hours. The cerebellum was isolated and weighed.

The cerebellar vermis from each animal was then embedded in paraffin and cut on a rotating microtome into 4µm-thick sections in the sagittal plane. Approximately every 20th section was mounted onto glass slides and stained with H&E. The midsagittal section from the cerebellar vermis of each animal was selected for analysis. This section was identified as the one in which the gap between lobules I and X was minimal, where the white matter was most narrow, and where deep cerebellar nuclei were absent (Bonthius et al., 2002).

Neuronal quantification

The total number of cerebellar Purkinje cells per section was quantified by manually counting Purkinje cell bodies in each section, while viewing the section under a microscope with a 40× objective. Purkinje cell bodies were included in the counts only if they contained a clearly identifiable cell nucleus.

The total number of cerebellar granule cells per section was also estimated. This was accomplished by multiplying the area of the granule cell layer by the density of granule cells within that layer. A microscope was coupled with a video camera to project an image onto a computer screen. The area of the granule cell layer was determined by digitally photographing each cerebellar vermis at low power, then using Image J software (Schneider et al., 2012) to outline the granule cell layer and measure its area. To determine the density of granule cells, an image of the cerebellar granule cell layer was projected at a higher power onto a computer screen. Cell densities were determined at 10 sites for each section. This was accomplished through the use of a 40u × 40u counting box, in which the number of cells within the box, but not touching the exclusion lines, were counted with the aid of the point counting program of Image J software. The sites for analysis within the granule cell layer were selected in a systematically random fashion by moving the microscope stage in a raster pattern with an x,y step of 650u × 450u. This assured that all sites within the granule cell layer were equally likely to be sampled. The overall density of granule cells for each section was determined by calculating the mean density from each of the 10 sampled areas.

Blood alcohol concentrations

Separate groups of animals were used to determine blood alcohol concentrations (BACs). Pups (n=5–6 per treatment and genotype) received alcohol (2.2, or 4.4 mg/g) daily over PD 4–8. BACs were determined four times for each pup from tail blood samples (20 ul each). The first blood sample was obtained 30 min following injection of the alcohol dose on PD 4. This time was chosen because it approximates the maximum BAC in mouse pups receiving IP injections of alcohol (Bonthius et al., 2002). The second blood sample was obtained the following morning, just prior to the next alcohol administration. This trough BAC reflected the extent to which the pups had cleared the alcohol dose administered the previous day. A second set of blood samples was obtained at the same time points following the PD 8 alcohol injection. Comparison of the BACs on PD 8 with those on PD 4 elucidated possible developmental changes in alcohol pharmacokinetics in both genotypes (Kelly et al., 1987). BACs were measured using an alcohol determination kit (Sigma, St. Louis, MO, USA) according to the manufacturer's directions.

Statistical analyses

Body weights and cerebellar weights were analyzed separately by analysis of variance (ANOVA). BACs (maximum and trough on PD 4 and PD 8) were analyzed by repeated measures ANOVA with genotype and alcohol treatment as the between-subjects factors and time-of-sampling as the within-subjects (repeated measure) factor.

For the balance beam data, the times for the two trials for each of the beams were first averaged. These averaged values were then analyzed by repeated measures ANOVA with

genotype, treatment (alcohol dose), and sex as the between-subjects factors and with the various beam shapes and sizes (large round, medium round, small round, large square, medium square, and small square) as the within-subjects (repeated measure) factors.

The rotarod data were analyzed by repeated measures ANOVA with genotype, treatment, and sex as the between-subjects factors and day of testing (days 1–4) as the within-subjects (repeated measure) factors. The total amount of time on the rotarod (over days 1–4) was also summed, and the values were analyzed by three-way ANOVA, with genotype, treatment, and sex as the between-subjects factors.

Purkinje cell number and granule cell number were analyzed together by three-way multivariate analysis of variance (MANOVA), with genotype, treatment, and sex as the between-subjects factors. To determine whether alcohol's effect on granule cell number was due to a change in granule cell density or to a change in the area of the granule cell layer, the cell densities and granule cell layer areas were analyzed by MANOVA.

The effect of alcohol on neuronal number for each of the cell populations (Purkinje cells and granule cells) was also expressed as a percent reduction from control. For the alcohol-exposed animals, this percent reduction from control is a direct measure of alcohol-induced microencephaly and cell loss (Bonthius and West, 1990). The percent reduction was calculated by subtracting the number of surviving neurons in each cell population in each alcohol-exposed mouse from the corresponding mean control value of the same cell population in mice of the same genotype, then dividing by the mean control value and multiplying by 100. These alcohol-induced percent cell losses for both of the cell populations were analyzed together by three-way MANOVA, with genotype, treatment group (2.2 and 4.4 mg/g), and sex as the fixed factors and with percent reduction in each cell population as the dependent variables. Similar alcohol-induced changes were calculated and analyzed for cerebellar weight.

Alcohol-induced changes were similarly calculated and analyzed for the behavioral data (total time on the rotarod, time on the rotarod by day, and time to traverse each balance beam). These alcohol-induced percent changes allowed visualization and analysis of the extent to which alcohol altered behavioral performance differently in the two genotypes. A significant main effect of genotype in this analysis would indicate that alcohol's effect on behavior depended on genotype.

For all of the analyses of variance, the components of the F values are reported as F(hypothesis degrees of freedom, error degrees of freedom). For all of the post-hoc analyses in this study, specific between-group comparisons were conducted with Bonferroni adjustments for multiple comparisons.

To determine the relationships among cell numbers and behavioral performances, correlational analyses were conducted between each of the quantified cell populations (Purkinje cells and granule cells) and the most relevant overall measure of performance on each behavioral test (total time on the rotarod and time to traverse the medium width square rod for the balance beam test). For these correlational analyses, Pearson correlation coefficients were calculated for each genotype (wild type and nNOS^{-/-} separately) and for

all mice in the study (wild type and nNOS^{-/-} combined). Correlations were considered significant at the 0.05 level and below.

Results

Blood alcohol concentrations were equivalent in nNOS^{-/-} and wild type mice

The maximum and trough BACs following alcohol administration on PD 4 and PD 8 are shown in Table 1. The acute administration of a daily bolus of alcohol resulted in high BACs, followed by low troughs that were at or near zero by the following morning. These fluctuations in BAC were reflected by a significant effect of time-of-sampling in the repeated measures analysis [$F(3,17) = 796.4$; $p < 0.001$]. For all groups, the maximum BAC was significantly higher than the trough ($p < 0.05$). The maximum BACs were dose-dependent, as reflected by a significant effect of treatment [$F(1,19) = 537.6$; $p < 0.001$]. In particular, for both genotypes on both PD 4 and PD 8, the maximum BAC following the high dose (4.4 mg/g/day) was significantly higher than the maximum BAC following the low dose (2.2 mg/g/day).

For all groups, the maximum and trough BACs on PD 8 were similar to the maximum and trough BACs, respectively, on PD 4. Within individual treatment groups and genotypes, there were no significant differences between the Day 4 values and the corresponding Day 8 values, indicating that there were no substantial developmental changes in alcohol pharmacokinetics between those two ages for either genotype.

The values and patterns of BAC were very similar for the nNOS^{-/-} and wild type mice. For both genotypes, the 2.2 mg/g alcohol dose resulted in maximum BACs of approximately 150 mg/dl, while the 4.4 mg/g dose resulted in maximum BACs of approximately 320 mg/dl. By the following morning, the mice of both genotypes and both alcohol doses had either cleared, or nearly cleared, the alcohol administered the previous day. There were no significant differences between wild type and nNOS^{-/-} mice in maximum or trough BAC on either day of testing. Thus, absence of nNOS gene function did not affect BAC profiles.

Alcohol impaired balance beam performance to a greater extent in nNOS^{-/-} mice than in wild type mice

The times required for the mice to traverse the round and square beams of different diameters and widths are shown in Figure 1. Because there were no main or interactive effects of sex, the data for the two sexes was combined. Greater amounts of time were required for the mice to traverse the smaller diameter and width balance beams than the larger ones. This was especially true for the mice that had been previously treated with alcohol. This finding that the time required to traverse the beams differed among the beams themselves was reflected by a significant effect of beam in the analysis [$F(5,75) = 49.25$; $p < 0.01$].

In the absence of alcohol, the nNOS^{-/-} mice and wild type mice performed similarly to each other. The nNOS^{-/-} mouse and wild type mouse performances did not differ significantly from each other on any of the beam shapes or sizes. Thus, absence of nNOS gene function alone did not affect performance on the balance beam test.

Alcohol impaired performance on the balance beam test for both the wild type and nNOS^{-/-} mice. For both genotypes and for most of the beams, the alcohol-treated mice required more time to traverse the beams than did the no-alcohol controls. This finding of alcohol-induced impairment on beam walking was confirmed by a significant effect of treatment group [$F(2, 1320.5) = 30.2$; $p < 0.001$]. The effect of alcohol was dose-related. In almost all cases, for both the wild type and the nNOS^{-/-} mice, the animals that received the high alcohol dose (4.4 mg/g) required more time to traverse the beam than those that received the low alcohol dose (2.2 mg/g), who, in turn, required more time than the no-alcohol controls.

While alcohol impaired performance for both the wild type and nNOS^{-/-} mice, alcohol affected the nNOS^{-/-} mice more severely than the wild type. This can best be appreciated in Figure 2, which demonstrates the alcohol-induced changes in performance for both genotypes. For almost all beam shapes and sizes, alcohol-induced changes in performance were significantly greater in the nNOS^{-/-} mice than in wild type. Thus, absence of nNOS gene function worsened alcohol-induced deficits in balance beam performance.

Alcohol impaired rotarod performance to a greater extent in nNOS^{-/-} mice than in wild type mice

Time spent on the rotarod for mice of the different genotypes and alcohol treatments on each of the four testing days is shown in Figure 3A. In general, average times spent on the rotarod were greater on days 3 and 4 than on days 1 and 2. This general improvement in rotarod performance with practice was confirmed by a significant effect of day-of-testing in the analysis [$F(3,77) = 27.98$; $p < 0.001$].

Genetic lack of nNOS (in the absence of alcohol) did not affect performance on the rotarod. On each day of testing, performance by the no-alcohol nNOS^{-/-} mice was very similar to that of the no-alcohol wild type mice.

Alcohol impaired rotarod performance for mice of both genotypes. On each of the four days of testing, mice of one or both genotypes that were exposed to alcohol performed more poorly than unexposed mice. Furthermore, alcohol's deleterious effect was dose-related, as the mice of both genotypes that were exposed to the high alcohol dose performed more poorly on most testing days than the low-dose or no-alcohol groups. This worsening of performance by alcohol on the daily performances was confirmed by a significant effect of treatment group [$F(2,79) = 9.62$; $p < 0.001$]. The dose-related effect of alcohol on rotarod performance can most easily be appreciated by examining total time on the rotarod (figure 3B). For both the wild type mice and the nNOS^{-/-} mice, as the dose of alcohol increased, the time spent on the rotarod decreased. The differences were statistically significant, however, only for the nNOS^{-/-} mice. This alcohol-induced diminishment in total time spent on the rotarod was confirmed by a significant effect of alcohol treatment group [$F(2, 79) = 9.618$; $p < 0.001$].

While alcohol impaired rotarod performance for mice of both genotypes, the effect was more severe in the nNOS^{-/-} mice than in the wild type mice. As shown in Figure 3C, at both the low alcohol dose (2.2 mg/g) and the high alcohol dose (4.4 mg/g), the alcohol-induced reductions in total time on the rotarod were greater for nNOS^{-/-} mice than for wild

type mice. This greater vulnerability of the nNOS^{-/-} mice to alcohol-induced reductions in total rotarod time was confirmed by a significant effect of genotype [$F(1, 53) = 6.285$; $p < 0.05$]. Figure 3D shows that the increased alcohol effect on the nNOS^{-/-} mice was not confined to a single day, but was a generalized effect that occurred on almost all testing days at both the low and high alcohol concentrations. Thus, as was true for the balance beam test, rotarod performance was impaired by alcohol, and the effect was substantially worse in the nNOS^{-/-} mice than in wild type.

Alcohol-induced reduction in cerebellar weight was more severe in nNOS^{-/-} mice than in wild type mice

The body and cerebellar weights of the nNOS^{-/-} and wild type mice are shown in Table 2. For both genotypes and for all alcohol doses, the male mice had greater body weights than the female mice. This larger size of the males was reflected by a significant effect of sex in the analysis of body weight [$F(1,79) = 31.64$; $p < 0.001$]. For all treatment groups and for both sexes, the wild type mice were larger than the nNOS^{-/-} mice. This growth restricting effect in the nNOS^{-/-} mice was confirmed by a significant effect of genotype in the analysis of body weight [$F(1,79) = 7.32$; $p < 0.005$]. Alcohol, however, did not affect body weight in either of the genotypes or for either of the sexes. Thus, there were no significant main or interactive effects of alcohol on body size.

As was true of body weights, cerebellar weights were likewise significantly lower in females than in males [$F(1,79) = 8.62$; $p < 0.005$] and significantly lower in nNOS^{-/-} than in wild type [$F(1,79) = 78.9$; $p < 0.001$]. However, unlike body weights, cerebellar weights were significantly affected by alcohol treatment [$F(2,79) = 12.54$; $p < 0.001$]. For male and female mice of both genotypes, exposure to alcohol significantly reduced cerebellar weights in a dose-dependent fashion.

The severity of alcohol's effect on cerebellar weight was not equal in the two genotypes, however. In particular, alcohol restricted cerebellar weight to a significantly greater extent in the nNOS^{-/-} mice than in wild type for both the low and high alcohol doses. This greater vulnerability of the nNOS^{-/-} mice was confirmed by a significant effect of genotype in the ANOVA of alcohol-induced changes in cerebellar weight [$F(1,53) = 7.89$; $p < 0.005$].

Alcohol-induced neuronal losses were more severe in nNOS^{-/-} mice than in wild type

The number of Purkinje cells and granule cells for both genotypes and all three treatment groups is shown in Figure 4. Although female mice of both genotypes tended to have slightly fewer Purkinje cells and granule cells than their male counterparts (data not shown), there were no statistically significant main or interactive effects of sex. Therefore, data for the two sexes was combined in Figure 4.

In the absence of alcohol, the nNOS^{-/-} mice had fewer Purkinje cells (Figure 4A) and granule cells (Figure 4B) than the wild type mice did. Specifically, the nNOS^{-/-} mice had approximately 17% fewer Purkinje cells and 11% fewer granule cells. This reduction in the number of Purkinje cells and granule cells in the mutant mice was confirmed by a significant effect of genotype on cell number in the MANOVA [$F(1, 79) = 117.9$; $p < 0.01$].

Exposure to alcohol reduced the number of Purkinje cells and granule cells in both the wild type and nNOS^{-/-} mice. This alcohol-induced loss of neurons was confirmed by a significant effect of alcohol treatment in the MANOVA [$F(4, 156) = 9.49$; $p < 0.001$]. This effect was dose-related, as the number of neurons in both cell populations and for both genotypes declined as the dose of alcohol increased (Figure 4 A&B). For the wild type mice, alcohol-induced neuronal losses were statistically significant only for the high dose of alcohol. In contrast, for the nNOS^{-/-} mice, neuronal losses for both cell populations were statistically significant for both the low and the high alcohol doses.

While alcohol adversely affected neuronal number for both genotypes, the magnitude of the effect was more severe in the nNOS^{-/-} mice than in wild type. This is most evident in Figure 4C, which depicts the percent cell losses induced by alcohol for both cell populations in the two genotypes. In the analysis of alcohol-induced cell losses, there was a significant effect of genotype [$F(2, 52) = 10.70$; $p < 0.001$], demonstrating that the effect of alcohol on neuronal cell loss depended on genotype. For both the Purkinje cells and the granule cells, at both the low dose and high dose of alcohol, percent cell losses were significantly worse in the nNOS^{-/-} mice than in wild type. The degree to which the nNOS^{-/-} mice were more vulnerable than wild type was substantial. For both cell populations and at both alcohol doses, cell deficits were at least twice as large for the nNOS^{-/-} mice than for wild type. For example, the high dose of alcohol reduced Purkinje cell number by 7.6% in wild type mice, but by 18.2% in nNOS^{-/-} mice. Thus, for both cerebellar Purkinje cells and granule cells, neuronal deficits were more severe in mice genetically deficient for nNOS than for wild type mice.

Alcohol reduced cerebellar size to a greater extent in nNOS^{-/-} mice than in wild type mice

Representative cross sections of the cerebellar vermis from wild type and nNOS^{-/-} mice are shown in Figure 5 A–D. In the absence of alcohol, the overall morphology and cytoarchitecture of the cerebellar vermis was normal in the nNOS^{-/-} mice. However, even in the absence of alcohol, the size of the cerebellum was smaller in the nNOS^{-/-} mice than in wild type. This reduction in cerebellar size in the nNOS^{-/-} mice was confirmed by a significant effect of genotype in the ANOVA of cerebellar cross sectional areas [$F(1, 79) = 56.1$; $p < 0.001$]. Thus, absence of the nNOS gene did lead to a reduction in cerebellar size.

Alcohol reduced the cerebellar cross sectional area in both wild type and nNOS^{-/-} mice (Figure 5E). This effect, which was dose-related, was confirmed by a significant effect of alcohol treatment in the ANOVA of cerebellar cross sectional areas [$F(2, 79) = 4.53$; $p < 0.05$]. Note, however, that this alcohol effect was more evident in the nNOS^{-/-} mice than in wild type and was statistically significant only in the nNOS^{-/-} mice. This greater vulnerability of the nNOS^{-/-} mice to alcohol-induced reductions in cerebellar size (Figure 5F) was confirmed by a significant effect of genotype in the analysis of alcohol-induced changes in cross section [$F(1, 53) = 5.39$; $p < 0.05$].

Behavior scores were highly correlated with neuron numbers for nNOS^{-/-} mice, but not for wild type mice

Because the behavioral data and neuronal cell count data were derived from the same animals in this study, it was possible to assess the degree to which the behavioral performances were correlated with neuronal number (Table 3). When all of the mice in the study (wild type and nNOS^{-/-}) were considered together, significant correlations emerged between both of the chosen behavioral measures (total time on the rotarod and time to traverse the medium square balance beam) and both of the cell populations examined (cerebellar granule cells and Purkinje cells). When only the nNOS^{-/-} mice were considered, most of these correlations remained significant. In contrast, when only the wild type mice were considered, all of the correlations became statistically insignificant. Thus, for the nNOS^{-/-} mice, there were strong relationships among neuronal losses and behavioral deficits, and these relationships were absent in wild type mice. An example is shown in Figure 6, in which there is a strong correlation between Purkinje cell number and performance on the balance beam for the nNOS^{-/-} mice, but lack of a significant correlation on these same parameters for the wild type mice.

Discussion

This study revealed three important new findings. First, genetic deficiency of nNOS worsened alcohol-induced performance deficits on cerebellum-mediated behavioral tests. On both the balance beam and the rotarod, alcohol impaired performance to a greater extent in nNOS^{-/-} mice than in wild type. Second, genetic deficiency of nNOS worsened alcohol-induced anatomical deficits in the cerebellum. In adult mice administered alcohol during early neonatal life, cerebellar weights, cross sectional areas, and neuronal numbers were all more severely decreased in the nNOS^{-/-} mice than in the wild type mice. Third, alcohol-induced deficits in neuronal number were correlated with behavioral deficits in nNOS^{-/-} mice but not in wild type. Cerebellar Purkinje cell losses and granule cell losses due to alcohol were more severe in mice genetically deficient for nNOS than in wild type, and these worsened cell losses were tied to worsened performances on the rotarod and balance beam tests. Thus, nNOS is the first gene identified whose mutation exacerbates the behavioral deficits of developmental alcohol exposure, and this effect appears to be mediated, at least in part, through worsened neuronal losses.

It has been known for many decades that the cerebellum plays an important role in motor activities, including coordination of movements, balance, gait, and postural control. Recent studies have demonstrated that the cerebellum has functions beyond motor control, as it is also involved in cognitive tasks, including verbal fluency, working memory, source memory, and executive function (Schlosser et al., 1998; Appollonio et al., Fiez et al., 1996). As a result, disorders of the cerebellum can impair a wide range of behaviors.

The cerebellum is a major target of alcohol teratogenesis. Autopsy studies of children with FAS have revealed cerebellar dysgenesis, heterotopia, and hypoplasia (Roebuck et al., 1998c), and MRI studies of children prenatally exposed to alcohol have revealed substantial reductions in the volumes of the cerebellar hemispheres and vermis (Autti-Ramo et al., 2002; Mattson et al., 1992; Norman et al., 2009). Alcohol can affect not only the structure of

the developing cerebellum, but its function as well (Coffin et al., 2005). As a group, children and adults prenatally exposed to alcohol have longer reaction times, slower movements, problems generating isometric and isotonic forces, impaired bimanual coordination, and disturbances in postural balance (Connor et al., 2006; Nguyen et al., 2013a,b; Roebuck et al., 1998a, b). They also commonly have difficulties with cognitive processes, including memory, verbal learning, and executive function (Vaurio et al., 2011). All of these behaviors are mediated either principally or in part by the cerebellum.

While the autopsy, neuroimaging, and behavioral studies commonly reveal cerebellar pathology and dysfunction as a consequence of prenatal alcohol exposure, not all children or adults are equally affected. For some, the cerebellar signs and symptoms are much more prominent or less prominent than for others. Even among those with fetal alcohol syndrome, in which the brain pathology and dysfunction are most severe, not all previously exposed people have cerebellar involvement (Roebuck et al., 1998c; Peiffer et al., 1979; Wisniewski et al., 1983). This suggests that intrinsic or environmental factors, or both, are at work to make alcohol's effects on the cerebellum more severe in some people and less severe in others. Genetics is certainly one of these factors.

We have shown previously that mice genetically deficient for nNOS (nNOS^{-/-} mice) are more vulnerable to alcohol-induced cerebellar neuronal deficits (Bonthius et al., 2002). This study substantially expands our previous findings by demonstrating that these mice have worsened alcohol-induced cerebellar dysfunction in adulthood and that these behavioral deficits are tied to permanently worsened alcohol-induced neuronal deficits.

The two broad categories of mutations affecting exons are gain-of-function mutations and loss-of-function mutations. A gain-of-function mutation confers new or enhanced activity on its protein product, while a loss-of-function mutation reduces or abolishes a protein's function. To date, no gain-of-function mutation has been identified that exacerbates the behavioral teratology of alcohol, and only two loss-of-function mutations are known that can do so. As shown here, nNOS is one of these. The other is oxoguanine glycosylase 1 (OGG1), a DNA repair enzyme and biomarker of oxidative stress. Mice homozygous for a null OGG1 mutation are more impaired than wild type by alcohol on a passive avoidance test and have increased oxidatively damaged DNA (Miller-Pinsler et al., 2015).

Two other gene mutations have been discovered that can worsen alcohol's embryopathy, though their effects on behavior have not been studied. The first of these is platelet-derived growth factor alpha (pdgfra), a receptor tyrosine kinase that regulates cellular migration, proliferation, and survival. Mutation of pdgfra worsens the effects of alcohol in a zebra fish model of FASD by interfering with neural crest cell migration and survival (McCarthy et al., 2013). However, this interaction between pdgfra mutations and alcohol exposure results in greater craniofacial defects, not in worsened brain structure or function. The second is catalase, an anti-oxidative enzyme. Administration of alcohol to catalase-deficient pregnant mice leads to greater DNA oxidative damage and worsened head length restrictions and total malformations in the embryos (Miller et al., 2013). Whether these worsened effects are principally due to the embryo's genotype or to the mother's, however, is unclear. The relative contributions of genes like nNOS, pdgfra, and catalase may explain why

neurocognitive deficits are alcohol's dominant effects in some children, while craniofacial abnormalities are dominant in others.

Two additional genes have been identified whose mutation can affect alcohol's impact on brain development. However, mutations in these genes *lessen* alcohol's effects, rather than worsen it. In particular, tissue plasminogen activator (tpa) is an extracellular protease that cleaves inactive plasminogen to plasmin. Tpa can also directly interact with the NMDA receptor to induce apoptosis in the hippocampus, and alcohol can kill developing hippocampal neurons through this same mechanism. Homozygous mutation of the tpa gene ameliorates alcohol's teratogenic effects by reducing alcohol-induced neurodegeneration in the hippocampus (Noel et al., 2011). Bax is a second gene whose mutation can ameliorate alcohol-induced pathology. A member of the Bcl-2 family of proteins, Bax can act as a pro-apoptotic factor, as it triggers the release of cytochrome c from mitochondria and induces cell death. In the developing cerebral cortex, alcohol can cause widespread neurodegeneration by inducing apoptosis. This adverse effect of alcohol on the developing brain fails to occur in Bax-deficient mice (Young et al., 2003; Heaton et al. 2006).

The mechanism by which nNOS protects the developing brain against alcohol-induced toxicity is not entirely understood. However, some key mechanistic aspects are known. nNOS is an enzyme that catalyzes the production of nitric oxide (NO), a gaseous molecule involved in numerous signaling pathways (Charriaut-Marlangue et al., 2013). NO produces its protective effect against alcohol by activating a particular signaling pathway, the NO-cGMP-PKG pathway (Bonthius et al., 2004). To produce neuroprotection, PKG phosphorylates a variety of effector molecules, the identities of which are not all known. However, one target of PKG is the transcription factor, NF-kappa B. At least part of the NO-cGMP-PKG pathway's neuroprotective effect is produced through activation of NF-kappa B (Bonthius et al., 2008; 2009). The downstream genes regulated by NF-kappa B that protect against alcohol toxicity remain to be identified, but likely include neurotrophic and anti-apoptotic factors.

The vulnerability of developing neurons to alcohol-induced cell death depends strongly on blood alcohol concentration (BAC), with higher BACs leading to greater cell losses (Bonthius et al., 1988; 1990). Thus, the question arises whether the worsened effects of alcohol in the nNOS^{-/-} mice may have been due to strain-related alterations in alcohol pharmacokinetics. Indeed, in this study, BACs were slightly, but consistently higher in the nNOS^{-/-} mice than in wild type. However, the differences were small and statistically insignificant. NO has been linked to changes in the alcohol metabolic pathway. However, the nature of this linkage is that increased nitrite concentration *inhibits* aldehyde dehydrogenase (Moon et al., 2005). This suggests that, if nNOS has any effect on alcohol metabolism, it should impair it rather than stimulate it. Yet, nNOS is protective. Thus, we doubt that the mechanism underlying the protective effect of nNOS is related to changes in alcohol metabolism. However, in this study we have measured alcohol concentrations only in blood, leaving open the possibility that there are pharmacodynamic differences within the *brains* of these mouse strains.

The relationship between NO and neuronal survival is not a simple one. The results of the present study suggest that NO can promote neuronal survival against alcohol. Others have similarly found a pro-survival effect of NO against a diversity of toxic conditions, such as glucose deprivation, glutamate toxicity, and trophic factor deprivation (Barger et al., 1995; Farinelli et al., 1996; Thippeswamy and Morris, 1997). However, NO can also impair neuronal survival, as has been shown in models of ischemia, infectious diseases, inflammatory disorders, and excitotoxicity (Dawson and Dawson, 1996). Thus, NO can be protective or injurious, and its role in any particular context probably depends on many factors, including cell type, maturational state, NO concentration, nature of the disease, and other intracellular and extracellular conditions.

The source of NO may also be critically important. Three different enzymes are sources of NO. These include neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO derived from iNOS, often in response to infection or inflammation, can be present at very high concentrations that are cytotoxic to the cells producing it and to surrounding cells (Hibbs et al., 1988). However, NO from nNOS can also be injurious, as it can worsen the injury induced by hypoxia and some other insults. On the other hand, as shown here, NO from nNOS can also be cytoprotective. Thus, the relationships among the source and concentration of NO, and its ultimate effects are multilayered and complex.

Nitric oxide can react with reactive oxygen species to form peroxynitrite, a reactive molecular species that can damage macromolecules. One way in which alcohol injures tissues to produce its teratogenic effects is by enhancing oxidative stress (Brocardo et al., 2011). Two other drugs that enhance oxidative stress to produce their teratogenic effects are phenytoin and benzo[a]pyrene. Both of these molecules are less developmentally toxic in iNOS knock-out mice (Kasapinovic et al., 2004). In contrast, as shown here, alcohol is more developmentally toxic in nNOS knock-out mice. Taken together, these studies show that genetic mutations of NOS enzymes can ameliorate some teratogenic agents, while they can worsen others, even when those agents share some of their teratogenic mechanisms.

We found that, even in the absence of alcohol, nNOS^{-/-} mice have smaller cerebellums and fewer Purkinje cells and granule cells than do wild type mice. This is the first time that genetic deficiency of nNOS alone has been linked to any neurohistological change. In our previous studies, we have found that nNOS^{-/-} mice generate and maintain normal complements of neurons within the cerebral cortex and hippocampus (Bonthius et al., 2006; Klein de Licon et al., 2009). Whether the reduction in cerebellar neurons in nNOS^{-/-} mice in this study was simply a scaling effect, due to the reduction in body size, or whether lack of nNOS gene function truly reduces cerebellar neuronal numbers remains to be determined.

Could the lack of nNOS explain why some human fetuses are so much more vulnerable than others to alcohol neurotoxicity? Complete genetic deficiency for nNOS has not been described in humans. However, nNOS gene expression is developmentally regulated in humans and experimental animals, and large changes in its levels and locations of expression occur during gestation (Ohyu and Takashima, 1998; Santacana et al., 1998). Furthermore, the gene is complex in its structure and regulation (Hall et al., 1994). It produces multiple mRNA transcripts via a variety of mechanisms, including alternate

promoter usage, alternative splicing, cassette insertions and deletions, and varied sites for 3'-UTR cleavage and polyadenylation. Some of the resulting transcript differences affect their translation, stability, and amino acid sequence (Wang et al., 1999). Furthermore, the nNOS gene is differentially regulated in different cell types and at different stages of development. In addition, multiple polymorphisms of the nNOS gene exist and affect the susceptibility to a variety of diseases (Chung et al., 1996; Grasemann et al., 1999; Levecque et al., 2003; Sayitoglu et al., 2006). Thus, while all humans possess the nNOS gene, specific brain structures may lack its expression in a form and at a time when its presence could be important for protection against alcohol teratogenesis.

nNOS is the first identified gene whose mutation can worsen alcohol-induced behavioral deficits. It is likely that there are many genes yet to be discovered whose variants worsen or ameliorate alcohol-induced damage to developing brain. These genetic differences among individuals likely contribute largely to the "spectrum" in fetal alcohol spectrum disorder.

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Highlights

Mice with homozygous null mutation of the nNOS gene have increased fetal alcohol effects.

Knockout of the nNOS gene worsens alcohol-induced cerebellar behavior deficits.

Knockout of the nNOS gene worsens alcohol-induced cerebellar neuronal losses.

Worsened effects of alcohol in nNOS^{-/-} mice are not due to altered EtOH metabolism.

Figure 1. Alcohol impaired balance beam performance in wild type and nNOS^{-/-} mice
The balance beam test consisted of one-meter beams of wooden dowels with a goal box on one end. The beams were either round or square and were one of three widths – small, medium, or large. Time required to traverse the 80-cm central portion of the beam was measured for each test trial. For the square beams (A) and for the round beams (B), alcohol worsened performance for both the wild type and nNOS^{-/-} mice. In the absence of alcohol, performance by the nNOS^{-/-} mice and wild type mice were similar.

* = significantly different from mice of the same genotype receiving 0.0 mg/g/day EtOH ($p < 0.05$).

= significantly different from mice of the same genotype receiving 2.2 mg/g/day EtOH ($p < 0.05$).

Figure 2. Alcohol worsened balance beam performance to a greater extent in nNOS^{-/-} mice than in wild type mice

For the square beams (A) and the round beams (B), alcohol-induced changes in the length of time required to traverse the balance beams were greater in the nNOS^{-/-} mice than in wild type mice. The only beam on which alcohol-exposed wild type mice and nNOS^{-/-} mice performed similarly was the small round beam. On all of the other beam shapes and sizes, alcohol impaired performance more severely in the nNOS^{-/-} mice than in wild type. This greater sensitivity to the adverse effects of alcohol among the nNOS^{-/-} mice was evident at both the low dose of alcohol (2.2 mg/g/day) and high dose of alcohol (4.4 mg/g/day).

* = significantly different from wild type mice administered the same daily dose of alcohol ($p < 0.05$).

= significantly different from mice of the same genotype administered the low dose of alcohol (2.2 mg/g/day) ($p < 0.05$).

Figure 3. Alcohol worsened rotarod performance to a greater extent in nNOS^{-/-} mice than in wild type mice

Wild type and nNOS^{-/-} mice were tested on an accelerating rotarod on each of four consecutive days. Each trial lasted a maximum of 240 seconds. The time at which the mice fell from the rotating rod was measured.

A. Alcohol impaired rotarod performance for both the wild type mice and the nNOS^{-/-} mice in dose-related patterns. * = significantly different from the 0.0 mg/g/day alcohol group of the same genotype ($p < 0.05$). ** = significantly different from the 0.0 and 2.2 mg/g/day alcohol groups of the same genotype ($p < 0.05$).

B. The duration of time that the mice remained on the rotarod on each of the four testing days was summed to yield a total time on the rotarod. Alcohol significantly reduced total time on the rotarod for the nNOS^{-/-} mice, but not for the wild type mice. * = significantly different from the 0.0 mg/g/day alcohol group of the same genotype ($p < 0.05$).

C. On most of the testing days, and for both doses of alcohol, the alcohol treatments significantly worsened rotarod performance to a greater extent in the nNOS^{-/-} mice than in wild type mice. * = significantly different from the wild type group administered the same dose of alcohol and tested on the same testing day ($p < 0.05$).

D. Alcohol-induced changes in total rotarod time were more severe in the nNOS^{-/-} mice than in the wild type mice. * = significantly different from the wild type group administered the same alcohol dose ($p < 0.05$). # = significantly different from mice of the same genotype administered the low dose of alcohol ($p < 0.05$).

Figure 4. Alcohol-induced neuronal losses were more severe in nNOS^{-/-} mice than in wild type mice

The number of Purkinje cells and granule cells was determined in single sections of 4 μ m-thickness through the midline vermis. A. In the absence of alcohol, the number of Purkinje cells was lower in nNOS^{-/-} mice than in wild type mice. Alcohol reduced the number of Purkinje cells in wild type mice and in nNOS^{-/-} mice. @ = significantly different from the 0.0 mg/g/day alcohol wild type mice ($p < 0.05$). * = significantly different from the 0.0 mg/g/day alcohol group of the same genotype ($p < 0.05$). B. The same patterns that emerged for Purkinje cells also occurred in granule cells. In the absence of alcohol, the number of

granule cells was lower in nNOS^{-/-} mice than in wild type mice. Alcohol reduced the number of granule cells in wild type mice and in nNOS^{-/-} mice. @ = significantly different from the 0.0 mg/g/day alcohol wild type mice ($p < 0.05$). * = significantly different from the 0.0 mg/g/day alcohol group of the same genotype. C. Alcohol-induced losses of Purkinje cells and granule cells were more severe in nNOS^{-/-} mice than in wild type mice. * = Significantly different from wild type mice of the same alcohol treatment group. # = Significantly different from mice of the same genotype receiving the 2.2 mg/g/day alcohol treatment.

Figure 5. Alcohol reduced the cerebellar cross sectional area to a greater extent in nNOS^{-/-} mice than in wild type mice

A–D. Photomicrographs reveal the differential effects of alcohol on the cerebellar vermis of nNOS^{-/-} and wild type mice. Pictured here are Nissl-stained 4- μ m-thick sections through the cerebellar vermis of young adult mice. Even in the absence of alcohol, the cerebellar size of the nNOS^{-/-} mouse (C) is notably smaller than the wild type mouse (A). Following exposure to the high dose of alcohol (4.4 mg/g/day), the cross sectional profile of the wild type mouse is minimally affected (B). In contrast, the cross sectional profile of the nNOS^{-/-} mouse is markedly reduced in size (D). Measurements of cross sectional areas (E) revealed

that, even in the absence of alcohol, the size of the cerebellar vermis was lower in nNOS^{-/-} mice than in wild type. Exposure to alcohol significantly reduced the cerebellar cross sectional area in the nNOS^{-/-} mice, but not in wild type mice. @ = significantly different from the 0.0 mg/g/day alcohol wild type group. * = significantly different from the 0.0 mg/g/day alcohol group of the same genotype. F. Alcohol-induced changes in cerebellar cross sectional areas were more severe in nNOS^{-/-} mice than in wild type mice. * = significantly different from wild type mice administered the same alcohol dose ($p < 0.05$). # = significantly different from mice of the same genotype administered the low dose (2.2 mg/g/day) of alcohol. Magnification bar represents 1mm in A, B, C, and D.

Figure 6. Balance beam performance was correlated with Purkinje cell number in nNOS^{-/-} mice, but not in wild type

Because the behavioral data and cell count data were derived from a single set of animals, it was possible to examine whether a relationship exists between these variables. Shown here are the times (in seconds) required to traverse the medium square balance beam (y-axis) plotted as a function of the number of Purkinje cells per section (x-axis). In the wild type mice (A), there was only a weak relationship between cell number and performance, with a correlation coefficient of -0.029 , which was not statistically significant. In contrast, in the nNOS^{-/-} mice (B), there was a much stronger relationship between performance and cell number, with a correlation coefficient of -0.423 , which was highly statistically significant. Note that the correlation coefficients are negative, reflecting the fact that the greater the number of surviving Purkinje cells, the less time was required to cross the beam.

Table 1

Blood alcohol concentrations (mg/dl)[#].

Genotype	Alcohol Dose (mg/g/day)	Day 4		Day 8	
		maximum	trough	maximum	trough
Wild type	2.2	151.1 ± 8.7	5.4 ± 1.9	144.4 ± 5.4	6.4 ± 1.8
	4.4	324.7 ± 12.2 *	7.6 ± 1.4	317.1 ± 8.4 *	6.9 ± 2.1
mNOS ^{-/-}	2.2	157.8 ± 6.0	5.1 ± 1.0	149.7 ± 3.8	5.6 ± 1.8
	4.4	345.3 ± 11.5 *	8.3 ± 2.2	330.8 ± 12.9 *	7.8 ± 0.9

[#] Values are means ± SEM.

* = Significantly greater maximum BAC in the 4.4 group than in the 2.2 group for mice of the same genotype.

Table 2

Effect of alcohol on body and cerebellar weights in nNOS^{-/-} and wild type mice.[@]

	Wild type mice						nNOS ^{-/-} mice					
	0.0		2.2		4.4		0.0		2.2		4.4	
	M	F	M	F	M	F	M	F	M	F	M	F
Body Weight (g)	32.5 ± 1.1	28.0 ± 0.8	32.7 ± 2.2	28.2 ± 1.4	33.4 ± 1.8	26.6 ± 1.8	30.7 ± 1.2	25.1 ± 1.2	30.3 ± 1.6	25.6 ± 1.8	31.0 ± 1.9	25.0 ± 1.3
Cerebellar Weight (mg)	65.3 ± 4.0	61.3 ± 2.2	66.0 ± 2.5	58.5 ± 1.8	58.9 ± 1.4	53.9 ± 2.5	55.0 ± 1.1 [*]	52.3 ± 2.3 [*]	50.0 ± 1.8 [*]	47.1 ± 2.3 [*]	45.0 ± 1.3 [*]	43.5 ± 2.9 [*]
Alcohol-Induced Change in Cerebellar weight (%)	NA		-1.7 ± 2.5		-10.9 ± 2.3 [#]		NA		-9.6 ± 2.5 ^{**}		-17.6 ± 2.8 ^{**#}	

[@] Values are means ± SEM.

^{*} =Significantly different from wild type of the same sex and alcohol treatment (p<0.05).

^{**} =Significantly different from wild type of the same alcohol treatment (p<0.05).

[#] =Significantly different from the 2.2 mg/g/day treatment of the same genotype.

Table 3Correlation coefficients between behavioral performances and neuronal numbers¹.

Genotype	Behavioral Test ^{2,3}	Neuron number	
		Cerebellar Granule Cells	Cerebellar Purkinje Cells
Wild Type	Balance Beam	NS	NS
	Rotarod	NS	NS
nNOS ^{-/-}	Balance Beam	-0.336 *	-0.423 **
	Rotarod	NS	0.393
Wild Type and nNOS ^{-/-} combined	Balance Beam	-0.288 **	-0.255 *
	Rotarod	0.326 **	0.348 **

¹ Numbers are Pearson Correlation Coefficients.² For the Balance Beam Test, the time to traverse the medium square balance beam was analyzed. The negative correlation coefficients imply that, as the number of neurons increased, the time required to traverse the beam decreased.³ For the Rotarod Test, the total time spent on the rotarod, summed over four days, was analyzed. The positive correlation coefficients imply that, as the number of neurons increased, the time spent on the rotarod increased.

* significant at the 0.05 level.

** significant at the 0.01 level.

NS = not significant.