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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. 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.

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1. 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,

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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, 2004a,b). 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.

2. Materials and methods

2.1. 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-h 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.

2.2. 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

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