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The neuronal nitric oxide synthase (nNOS) gene and neuroprotection against alcohol toxicity

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Abstract

When a mother abuses alcohol during pregnancy, the offspring can suffer a myriad of abnormalities, collectively known as Fetal Alcohol Spectrum Disorder (FASD). Foremost among these abnormalities is central nervous system dysfunction, which commonly manifests itself as mental retardation, clumsiness, hyperactivity, and poor attention span. These behavior problems are due, in large part, to alcohol-induced neuronal losses in the developing fetal brain. However, not all fetuses are equally affected by maternal alcohol consumption during pregnancy. While some fetuses are severely affected and develop hallmarks of FASD later in life, others exhibit no evident neuropathology or behavioral abnormalities. This variation is likely due, at least in part, to differences in fetal genetics. This review focuses on one particular gene, neuronal nitric oxide synthase, whose mutation worsens alcohol-induced neuronal death, both *in vitro* and *in vivo*. In addition, ectopic expression of the nNOS gene protects neurons against alcohol toxicity. The gene encodes an enzyme that produces nitric oxide (NO), which facilitates the protective effects of neuronal growth factors and which underlies the ability of neurons to resist alcohol toxicity as they mature. Nitric oxide exerts its protective effects against alcohol via a specific signaling pathway, the NO-cGMP-PKG pathway. Pharmacologic manipulation of this pathway could be of therapeutic use in preventing or ameliorating FASD.

Keywords

Fetal Alcohol Spectrum Disorder; FASD; Nitric Oxide; nNOS; NFkB; ethanol; cerebellar granule neuron

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Introduction

Alcohol abuse during pregnancy can severely damage the fetal brain, causing a wide range of symptoms later in life, collectively known as the Fetal Alcohol Spectrum Disorder (FASD). Because of alcohol's toxic effects on the developing brain, children with FASD are often microcephalic and suffer from mental retardation, epilepsy, attention deficits, and behavioral disturbances (Olson et al., 1998; Riley et al., 2011). Despite publicity efforts and warnings from the Surgeon General's office, many women continue to drink alcohol during pregnancy and give birth to children with FASD. In fact, FASD is one of the leading causes of mental retardation in the United States and poses a major public health concern (May et al., 2014). The economic cost of FASD is estimated at several billion dollars per year in the United States (Lupton et al., 2004), while its human cost is staggering and incalculable.

Abnormalities in midface development and impairment of somatic growth are two hallmark features of FASD and are keys to its diagnosis (Hoyme et al., 2005). However, it is alcohol's effect on the developing *brain* that is key to its clinical impact. Alcohol can disrupt many aspects of brain development, including synapse formation, neurite extension, neuronal migration, neuronal differentiation, and many others. However, one of alcohol's most important adverse effects on the developing brain is neuronal death. Animal studies have shown that the developing brain is particularly susceptible to alcohol-induced neuronal death and that this loss of neurons plays an important role in the microencephaly that follows alcohol exposure (Bonthius and West, 1990; Ikonomidou et al., 2000). Neuronal death also plays a key role in the neurobehavioral deficits accompanying FASD, as alcohol-induced neuronal losses are highly correlated with learning deficits (Goodlett et al., 1992), impaired coordination (Thomas et al., 1998), and reduced seizure thresholds (Bonthius et al., 2001).

While alcohol can kill neurons in the developing brain, not all brain regions are equally vulnerable. Animal studies employing stereological neuronal counts or molecular markers of apoptosis have demonstrated that the cerebellum, hippocampus, olfactory bulbs, cerebral cortex, and basal ganglia are particularly vulnerable, while other brain regions are largely or completely spared (Bonthius et al., 1992; Bonthius and West, 1990; Olney et al., 2000). Neuroimaging studies in humans with FASD have largely confirmed these results (Autti-Ramo et al., 2002; Moore et al., 2014). Thus, the developing brain's vulnerability to alcohol toxicity is region-dependent.

Certain neuronal populations are vulnerable to alcohol toxicity during an early developmental period. However, those same neuronal populations become resistant to alcohol toxicity in adulthood. This phenomenon explains why a human fetus is so much more damaged by maternal alcohol consumption than is the mother, despite the fact that the fetal and maternal brains are exposed to very similar alcohol concentrations and durations (Chernoff, 1980). The finding that developing neurons transition from vulnerable to resistant with the passing of time gives rise to the concept of "temporal windows of vulnerability" to alcohol (Goodlett and Johnson, 1999). How neurons undergo this transition is not completely understood and remains a key question in FASD research.

We have begun to address this question by reasoning that the change in resistance over time is due either to changes within the neurons themselves, which are likely genetically programmed, or to changes in the extrinsic support of the neurons, which are likely growth factors. Therefore, we assessed how changes in neuro-developmental gene expression compare with changes in growth factor expression as the cerebellum transitions from alcohol-vulnerable to alcohol-resistant over time (Karacay et al., 2008). We found that the vulnerability of developing cerebellar neurons to alcohol toxicity declines in parallel with decreasing expression levels of genetic markers of immaturity, such as Math1 and Cyclin D2, and with increasing expression levels of genetic markers of neuronal maturation, such as GABA α -6 and Wnt-7a. In contrast, growth factors do not significantly change their expression levels during this transition period (Karacay et al., 2008). These results suggest that processes *intrinsic* to neuronal maturation, rather than rising levels of extrinsic growth factors, underlie maturation-dependent alcohol resistance. The identities of these intrinsic factors are unknown. However, as discussed below, one of the most important of them may be increasing expression levels of a particular gene, neuronal nitric oxide synthase (nNOS).

Genes and Alcohol Vulnerability

It has long been observed that the rate of alcohol metabolism differs among people of different ethnic origins (Suddendorf, 1989), varying as much as three- to four- fold from one group to another (Li et al., 2001). These differences in alcohol metabolism rates can manifest themselves not only in differences in the duration of alcohol's intoxicating effects, but also in qualitatively different responses to alcohol. The "alcohol flush reaction" (also known as the "Asian flush syndrome") is a condition in which a person flushes or develops skin blotches shortly after consuming alcoholic beverages. This condition is due to a genetically-determined altered pattern of alcohol metabolism and provides evidence that genetics play an important role in differential alcohol metabolism among individuals (Peng et al., 2014).

In humans, more than 90% of ingested alcohol is metabolized in the liver. Upon reaching the liver, alcohol is first metabolized to acetaldehyde. The principal enzyme responsible for this conversion is alcohol dehydrogenase (ADH). The resulting acetaldehyde is next metabolized to acetate, mainly through the action of aldehyde dehydrogenase (ALDH) (Quertemont, 2004). Acetate is then broken down into water and carbon dioxide.

Acetaldehyde is a toxic and reactive compound. In most people who have consumed alcohol, acetaldehyde is present for only a very short period, before it is metabolized to acetate, an essentially benign substance that is much less reactive. However, some individuals have forms of aldehyde dehydrogenase that are less efficient at metabolizing acetaldehyde. As a consequence, tissue levels of acetaldehyde rise to higher levels. These are the individuals who suffer from the alcohol flush reaction. Genetic studies show that the condition is associated with a certain form of the ALDH gene, the rs671 allele. This form of ALDH is carried by 30-50% of people of Chinese and Japanese origin, but rarely carried by members of other ethnic groups (Li et al., 2009). It is for this reason that the alcohol flush reaction is principally observed in people of Asian descent.

Just as there are polymorphisms for the aldehyde dehydrogenase (ALDH) gene, so there are also polymorphisms for alcohol dehydrogenase (ADH). These individual forms of ADH have different enzyme kinetics. As a result, following ingestion of alcohol, individuals can experience different blood alcohol concentrations. For example, the ADH1B2 and ADH1B3 alleles increase the rate of alcohol metabolism, while the ADH1B1 allele has a slower rate of alcohol metabolism. As a result, the blood alcohol concentration falls more rapidly in individuals with the ADH1B2 or ADH1B3 alleles than in those with ADH1B1 (Neumark et al., 2004; Thomasson et al., 1995).

Both alcohol and acetaldehyde are toxic for the embryo and fetus (Lee et al., 2005). Thus, it stands to reason that the more rapidly these harmful substances are cleared from the body, the less injurious the ingestion of alcohol will be. However, it is the mother and not the fetus that metabolizes the bulk of ingested alcohol (Chernoff, 1980). This suggests that the *mother's genotype* influences the outcome of alcohol consumption on the fetus during pregnancy (Warren and Li, 2005). In support of this notion, several studies have demonstrated that the ADH1B2 and ADH1B3 alleles are underrepresented among the mothers of children with FASD (Das et al., 2004; Jacobson et al., 2006; McCarver et al., 1997; Viljoen et al., 2001). This is consistent with the notion that the ADH1B2 and ADH1B3 alleles have protected the fetus, by rapidly clearing the alcohol, and made FASD less likely. However, it is unknown whether this underrepresentation is actually due to a more rapid clearance of alcohol or to a reduction in alcohol consumption by the mothers.

While prenatal alcohol consumption substantially damages some fetuses, others exposed to similar, or even greater, amounts of alcohol are not affected (Abel, 1995; Jacobson et al., 1998). These differences in outcome may be due, in part, to differences in the gestational timing of alcohol consumption, differences in the patterns in which expectant mothers consume alcohol, and differences in maternal health and nutrition (May et al., 2013). However, a great deal of the outcome differences is likely due to differences in *fetal genetics*. Individual fetuses probably possess specific genes or gene combinations that make them less or more vulnerable than others to alcohol's toxic effects.

The most convincing evidence that fetal genes play an important role in determining vulnerability to FASD has come from twin studies. Shortly after fetal alcohol syndrome was first described in 1973, a pair of dizygotic twins was reported, one of which had fetal alcohol syndrome, while the other did not (Christoffel and Salafsky, 1975). The twins had obviously been exposed to virtually identical blood alcohol profiles and other maternal factors *in utero*. A difference in fetal genetics was the most likely explanation for the incongruence. This prompted a study comparing dizygotic and monozygotic twins, which found a concordance rate for FASD of 100% among the monozygotic twins, but only 64% among the dizygotic twins (Streissguth and Dehaene, 1993). These findings strongly suggest that fetal genotype plays an important role in determining vulnerability to FASD (Gemma et al., 2007; Warren and Li, 2005). But what are the genes responsible for these vulnerability differences?

Because alcohol's principal target in producing its adverse effects in FASD is the fetal brain, it is likely that fetal genes involved in the development, function, and differentiation of

neurons play a role in determining fetal risk to FASD. Furthermore, because neuronal death is such an important component of FASD pathogenesis, it is likely that genes influencing neuronal survival are among the most important. Hence, in our attempt to identify specific genes of importance to FASD, we have focused on genes with neuroprotective properties. Identification of such genes is of paramount importance for understanding how alcohol creates its devastating effects and for development of therapeutic approaches for the amelioration of FASD.

NMDA-Mediated Neuroprotection Against alcohol Toxicity

Primary cultures of cerebellar granule cells (CGCs) are a major tool for studying alcohol's neuroteratogenic effects. These cultures are a valuable model system because (a) the cultured cells consist principally of neurons, which are likely the major target of alcohol in FASD, (b) the *in vitro* system allows experimental manipulation of alcohol concentrations and other extracellular factors, and (c) the cells are vulnerable to alcohol toxicity. Several studies, including our own, have demonstrated that alcohol induces substantial cell death in cerebellar granule cell cultures (Bonthius et al., 2004a; Bonthius et al., 2003; Bonthius et al., 2002; Karacay et al., 2007; Luo, 2012). When cerebellar granule cell cultures are exposed to pharmacologically relevant concentrations of alcohol (100–400 mg/dl), approximately 10–25% of the cells are killed. The precise mechanism by which alcohol kills cultured neurons is unknown, but we initially hypothesized that it might do so through excitotoxicity.

N-Methyl-D-aspartate (NMDA) is an amino acid derivative that can mimic the action of the neurotransmitter glutamate by specifically activating the glutamate NMDA receptor. Because activation of the NMDA receptor is excitatory, we initially hypothesized that NMDA would worsen alcohol-induced neuronal death in cerebellar granule cell cultures, as it would augment excitotoxicity. We found precisely the opposite result, however. Instead of worsening alcohol neurotoxicity, NMDA *protected* the cells against alcohol-induced cell death (Hutton-Kahrberg et al., 2004). Moreover, 2-amino-5-phosphonovalerate, APV, a specific antagonist of the NMDA receptor, abolished the protective effect of NMDA. These results demonstrated that NMDA has a neuroprotective effect against alcohol toxicity in cultures of cerebellar granule neurons, and that this effect is NMDA receptor specific (Hutton-Kahrberg et al., 2004). How NMDA exerts this protective effect was initially a mystery.

NMDA exerts its protective effect against alcohol toxicity by activating nNOS

Stimulation of the NMDA receptor by binding of its specific agonist to its NR2 subunit opens cation channels within the NMDA receptor that allow the passage of Ca^{2+} into the cell (Paoletti and Neyton, 2007). One important outcome of the Ca^{2+} influx is activation of the calcium-dependent neuronal nitric oxide synthase (nNOS) enzyme, which produces nitric oxide (NO) within neurons. Thus, the possibility arose that the mechanism of NMDA-mediated neuroprotection might involve the NO produced by nNOS. This idea predicts that exogenous NO will be neuroprotective, while agents that block the action of nNOS will be injurious. This is precisely what we found. Administration of DETA-NONOate, a molecule

that serves as an NO donor, protected the cultured cerebellar granule cells against alcohol toxicity to a degree very similar to that seen with NMDA. Conversely, L-NAME, a compound that blocks nNOS from producing NO, completely blocked the protective effects of NMDA. Thus, we found convincing evidence that the protective actions of NMDA against alcohol neurotoxicity were produced through the actions of a specific molecule: nitric oxide (Hutton-Kahrberg et al., 2004)

Nitric oxide is a gaseous molecule that can protect or injure tissues, depending on context.

Nitric oxide (NO) is a small gaseous molecule with multiple functions, including neurotransmission, regulation of vascular tone, and participation in several intracellular signaling pathways (Charriaut-Marlangue et al., 2013). While the discovery of NO dates back to the eighteenth century, it was not until the latter part of the twentieth century that its many physiological roles began to be discovered. Initially, nitrate-based compounds, such as nitroglycerine, were found to increase guanylyl cyclase activity. This activity was associated with an increased cGMP concentration and smooth muscle relaxation (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Marsh and Marsh, 2000). Both the increase in cGMP concentration and the muscle relaxation could be mimicked by NO-donors, such as nitroglycerin and exogenous NO (Arnold et al., 1977; Ignarro et al., 1981; Katsuki et al., 1977), thus strongly suggesting that release of NO was the underlying basis for these effects.

These discoveries prompted a burst of research on the physiology of NO, particularly in the 1980s and early 1990s. NO was named as the molecule of the year by *Science* magazine in 1992, and the Nobel Prize in Physiology or Medicine in 1998 was awarded for the discovery of NO's function in vasodilation. During this flurry of research focused on NO, the molecule was found to play an important role, not just in the vascular system, but in the central nervous system, as well. NO was discovered to act as a neurotransmitter and neuromodulator, and was found to play critical roles in normal brain function, including learning and memory (Garthwaite and Garthwaite, 1987; Garthwaite et al., 1988). NO was also identified as a pro-survival molecule that could protect a variety of cell types, including neurons, against a diversity of toxic conditions and insults, such as glucose deprivation, glutamate toxicity, and trophic factor deprivation (Barger et al., 1995; Farinelli et al., 1996) (Thippeswamy and Morris, 1997). Around the same time, NO generated at high concentrations by macrophages was found to have cytotoxic effects both on the surrounding cells and on the macrophages themselves (Hibbs et al., 1988). Furthermore, compelling evidence was found that NO can exacerbate cell death in multiple pathologic conditions, including ischemia, infectious diseases, inflammatory disorders, and excitotoxicity (Dawson and Dawson, 1996). Thus, it was discovered that NO can act as a two-edged sword, as it enhances neuronal survival in some circumstances, while it promotes neuronal death in others. Factors influencing whether NO is protective or injurious in any context include the cell type, maturational state, NO concentration, NO source, nature of the disease, and other intracellular and extracellular conditions (Contestabile et al., 2003).

In neurons, NO is synthesized by the neuronal nitric oxide synthase (nNOS) enzyme which converts the amino acid L-arginine into NO and citrulline (Dawson et al., 1998; Villalobo,

2006). The NO can act in a variety of ways, but one of its most prominent effects is stimulation of a particular signaling pathway, the NO-cGMP-PKG pathway. As shown in Figure 1, in this pathway, the NO stimulates soluble guanylyl cyclase (sGC), the enzyme that produces the cyclic nucleotide, cGMP. This raises intracellular levels of cGMP (Arnold et al., 1977; Friebe et al., 1998). Further downstream, cGMP activates cGMP-dependent protein kinase (PKG) (Lincoln et al., 1994), which, in turn, produces the pathway's biological effects by phosphorylating target proteins. We hypothesized that NO exerts its neuroprotective effects against alcohol toxicity through the NO-cGMP-PKG pathway (Bonthius et al., 2004a).

To test this hypothesis, we took advantage of a variety of pharmacological agents that specifically activate or inhibit the pathway at each of its various steps (Figure 1). We exposed cerebellar granule cell cultures to alcohol and conducted “block and rescue” experiments in which we determined the effect of these agents, alone or in combination, on alcohol-induced cell death (Bonthius et al., 2004a). We found that the protective effects of DETA-NONOate, which activates the NO-cGMP-PKG pathway at its first step by mimicking nNOS, could be completely blocked through the actions of LY83583, which blocks the pathway at its second step by inhibiting sGC. Similarly, the protective effects of 8-Br-cGMP, which activates the pathway at its second step by serving as an analog of cGMP, could be blocked by PKG inhibitor (Rp-8-pCPT-cGMPS), which blocks the pathway at its third step by inhibiting the kinase activity of PKG. Through a whole series of such experiments, we found that activation of the NO-cGMP-PKG pathway is protective, while inhibition of the pathway is injurious (Bonthius et al., 2004a). Furthermore, activation of the pathway downstream of a pathway inhibitor is protective, while inhibition of the pathway downstream of an activator is injurious. Taken together, these observations demonstrate that activation of the NO-cGMP-PKG pathway can protect developing neurons against alcohol toxicity and that NO exerts its neuroprotective effects via this pathway.

Several, but not all, endogenous growth factors utilize the NO-cGMP-PKG pathway to produce their neuroprotective effects against alcohol.

Through the use of cerebellar granule cell cultures, a variety of growth factors have been identified that can protect developing neurons against alcohol-induced cell death (Bhave et al., 1999; Luo et al., 1997). The mechanism by which they produce this protective effect has been unclear. Our studies of nitric oxide signaling have begun to shed some light on this issue (Bonthius et al., 2003). In cerebellar granule cell cultures exposed to alcohol, there are two separate processes leading to neuronal death. First, there is “background cell death.” Like most cultures of primary neurons, some of the cerebellar granule cells initially placed in culture do not survive, even in the absence of alcohol. Second, there is “alcohol-induced cell death.” This is the death of neurons above the background level and is attributable to the toxic effects of alcohol on the cultures.

A survival-promoting agent may protect cells against one or both of these cell death processes. One can discern an agent's effect on each of these processes by initially plating equal numbers of cells into culture wells, then comparing cell numbers in the culture dishes with and without the agent, and in the presence and absence of alcohol. If an agent reduces

background cell death in alcohol-free cultures, then that agent has a “neurotrophic” effect. If an agent reduces ethanol-induced cell death, then the agent has a “neuroprotective” effect (Bonthius et al., 2003).

Using cerebellar granule cell cultures exposed to alcohol, we have found that individual endogenous growth factors can have neurotrophic effects, neuroprotective effects, both, or neither (Table 1). For example, basic fibroblast growth factor (FGF-2) reduces background cell death (a neurotrophic effect) and protects against alcohol-induced cell death (a neuroprotective effect), while epidermal growth factor (EGF) does not affect cell survival in the absence or presence of alcohol (neither a neurotrophic nor a neuroprotective effect). In addition, growth factors can be neurotrophic, but not neuroprotective, as is the case for insulin-like growth factor (IGF-1), or neuroprotective but not neurotrophic, like nerve growth factor (NGF) (Bonthius et al., 2003).

Using pharmacological inhibitors of the NO-cGMP-PKG pathway (figure 1), we investigated whether specific growth factors rely upon the pathway to signal their neurotrophic and neuroprotective effects (Bonthius et al., 2003). We reasoned that, if the survival-promoting effects of a growth factor can be abolished with an inhibitor of the NO-cGMP-PKG pathway, then that growth factor must rely upon the pathway to signal its effects.

As shown in Table 1, we found that several growth factors do utilize the NO-cGMP-PKG pathway to produce their survival promoting effects. For example, FGF-2 and NGF can each protect neurons against alcohol-induced cell death, but this neuroprotective effect is abolished when the NO-cGMP-PKG pathway is blocked at its first step with NAME, at its second step with LY83583, or at its third step with PKG inhibitor (Figure 1 and Table 1). Not all growth factors utilize the pathway, however. Brain-derived neurotrophic factor (BDNF) exerts both a neurotrophic and neuroprotective effect against alcohol, but neither of these survival-promoting effects can be blocked by inhibitors of the NO-cGMP-PKG pathway. Taken together, these studies suggest that there is an overlap in the signaling pathways for some, but not all, growth factors. In addition, the studies emphasize the importance of NO in growth factor-mediated neuronal protection in the face of alcohol (Bonthius et al., 2003).

Neurons lacking the nNOS gene are more vulnerable than wild type neurons to alcohol-induced cell death.

The strongest evidence for the importance of the NO-cGMP-PKG pathway in protecting neurons against alcohol-induced death has come from our studies employing cerebellar granule cells isolated from mice lacking a functional nNOS gene (Bonthius et al., 2008; Bonthius et al., 2006; Bonthius et al., 2002). We reasoned that, if the pathway is important for protecting neurons against alcohol toxicity, then neurons lacking nNOS should be especially vulnerable, since they cannot produce NO and, consequently, cannot utilize the NO-cGMP-PKG pathway for neuroprotection.

To test our hypothesis we compared rates of alcohol-induced cell death in cerebellar granule cell cultures derived from wild type mice and mice carrying a homozygous null mutation of the nNOS gene (nNOS^{-/-} mice) (Huang et al., 1993). We treated the cultures with increasing concentrations of alcohol (0, 100, 200, and 400 mg/dl) and found that alcohol reduced the number of surviving neurons in a dose-dependent manner in both genotypes (Bonthius et al., 2002). However, nNOS^{-/-} cells were substantially more severely affected than wild type cells at each of the three alcohol concentrations. Lack of nNOS not only affected the relative severity of the cell losses, but reduced the threshold concentration of alcohol necessary to induce cell death. Whereas the lowest alcohol concentration (100mg/dl) had no effect on cell survival in the wild type cultures, the same concentration significantly reduced cell survival in the nNOS^{-/-} cultures. At the higher concentrations (200 and 400mg/dl) cell survival was significantly lower in nNOS^{-/-} cultures than in wild type cultures (Bonthius et al., 2002).

To obtain further evidence that nNOS exerts a neuroprotective effect against alcohol toxicity, we carried out a rescue experiment using nNOS^{-/-} cerebellar granule cells and an nNOS expression vector (Karacay et al., 2007). We reasoned that, if delivery of nNOS cDNA into nNOS^{-/-} cells reduces their vulnerability to alcohol toxicity, then this neuroprotection must be a direct effect of nNOS expression. We transduced nNOS^{-/-} cerebellar granule cells with an adenoviral vector carrying an nNOS gene expression cassette (Ad5-nNOS) and assessed the alcohol vulnerability of the transduced cells. Alcohol induced substantial amounts of cell death in both the un-induced cells and the cells induced with an empty (control) vector. In contrast, when nNOS^{-/-} cells were infected with Ad5-nNOS, alcohol-induced cell death was eliminated. In other words, delivery of the nNOS gene into cells that originally lacked it converted the cells from alcohol-vulnerable to alcohol-resistant.

Although wild type cerebellar granule cells suffer less alcohol-induced cell death than nNOS^{-/-} cells, they still have some neuronal losses from alcohol. However, when we transduced the wild type cells, the over-expression of nNOS completely protected them against alcohol-induced cell death (Karacay et al., 2007). The results provide strong evidence that nNOS is a powerfully protective gene against alcohol-induced death. These experiments were the first of their kind, demonstrating that gene therapy approaches can be used to protect developing neurons against alcohol toxicity.

We found that the enhanced vulnerability of the nNOS^{-/-} cells to alcohol toxicity could be reversed, not only genetically, but pharmacologically as well (Bonthius et al., 2008). Since nNOS^{-/-} cells do not produce NO, they cannot activate the first step of the NO-cGMP-PKG pathway for neuroprotection. However, the remainder of the pathway should be available and functional, if that first step is bypassed. We tested this hypothesis by treating cultured cerebellar granule cells from wild type and nNOS^{-/-} mice with pharmacological activators and inhibitors of the NO-cGMP-PKG pathway downstream from nNOS (Figure 1). We found that DETA-NONOate, an NO donor that activates the pathway at its second step, is protective for both nNOS^{-/-} neurons and wild type. Importantly, in the presence of DETA-NONOate, the vulnerability of nNOS^{-/-} neurons diminished to a level equivalent to wild type. This same protection of the nNOS^{-/-} cells occurred when the pathway was activated

at its third step through the administration of 8-Br-cGMP (Bonthius et al., 2008). These results demonstrate that nNOS^{-/-} neurons are more vulnerable than wild type to alcohol toxicity and that their inability to activate the NO-cGMP-PKG pathway is the basis for their increased vulnerability.

The NO-cGMP-PKG pathway is essential for maturation-dependent alcohol resistance.

The maturational state of neurons plays an important role in determining their vulnerability to alcohol toxicity. In general, during the developmental period, as neurons become more mature, they become less vulnerable to alcohol-induced cell death (Goodlett and Johnson, 1999). This transition from alcohol-vulnerable to alcohol-resistant can occur rapidly. For example, we have shown that alcohol administration to mice over postnatal days 2-4 causes substantial reductions in neuronal number and in the size of the cerebellum. However, administration of equivalent doses of alcohol at the slightly later age of postnatal days 8-10 causes far less cerebellar injury (Karacay et al., 2008). This reduction in alcohol vulnerability with age is referred to as “maturation-dependent alcohol resistance.”

Cultured cerebellar granule cells also acquire maturation-dependent alcohol resistance. When the cultures are exposed to alcohol after just one day in vitro (1-DIV), a substantial proportion of cells die, thus demonstrating that the cells are highly vulnerable to alcohol toxicity at that stage. However, if the cells are exposed to alcohol just three days later, on the fourth day in vitro (4-DIV), then only a small proportion of the cells die. In fact alcohol exposure kills as many as 10-fold more cells in 1-DIV cultures than in 4-DIV cultures (Hutton-Kahrberg et al., 2004). The existence of maturation-dependent alcohol resistance in cultured neurons is valuable because it provides an excellent model system in which to investigate the molecular mechanism of this highly important phenomenon.

We hypothesized that maturation-dependent alcohol resistance is linked to the NO-cGMP-PKG pathway. This hypothesis predicts that 4-DIV cultures, which are normally resistant to alcohol-induced cell death, will become vulnerable to alcohol if the NO-cGMP-PKG pathway is blocked. Therefore, we treated 4-DIV cultures with pharmacological inhibitors of the pathway and determined their vulnerability to alcohol. L-NAME and LY83583, which inhibit the pathway at its first and second steps, respectively (Figure 1), converted 4-DIV cultures from alcohol-resistant to alcohol-sensitive. In fact, the magnitude of the alcohol-induced cell death in 4-DIV cultures treated with pathway inhibitors was similar to that observed in the alcohol-sensitive 1-DIV cultures (Bonthius et al., 2004a). Therefore, while becoming alcohol-resistant as they mature from 1-DIV to 4-DIV, cerebellar granule cell cultures revert to alcohol-vulnerable when the NO-cGMP-PKG pathway is disrupted. This provides strong evidence that maturation-dependent alcohol resistance is, at least in part, a function of the NO-cGMP-PKG pathway.

To further test the notion that maturation-dependent alcohol resistance is linked to nitric oxide, we employed cerebellar granule cell cultures lacking the nNOS gene. We reasoned that, if maturation-dependent alcohol resistance requires signaling through an NO-mediated pathway, then this form of alcohol resistance should be absent in nNOS^{-/-} neurons, since

they lack the enzyme to produce NO. Cultures from nNOS^{-/-} mice would remain sensitive to alcohol, even at 4-DIV, when wild type cultures demonstrate resistance. Indeed, this was the result we obtained. CGCs from wild type mice became resistant to alcohol-induced death as they underwent maturation in culture, whereas cells lacking nNOS remained sensitive (Bonthius et al., 2008). Taken together, these studies demonstrate that NO, via the NO-cGMP-PKG pathway, plays a key role in the ability of neurons to resist alcohol's toxic effects as they mature.

The precise mechanism by which the NO-cGMP-PKG pathway exerts this maturational effect is unknown. One possibility is that a component of the pathway is subject to developmental regulation and that its level of expression increases as neurons mature. Indeed, nNOS expression is developmentally regulated (Baader et al., 1997; Ciani et al., 2002). While nNOS expression in cerebellar granule cells is very low in 1-DIV cultures, its expression increases significantly between 3DIV and 5-DIV (Ciani et al., 2002). Thus, in cerebellar granule cell cultures, the developmental expression of nNOS precisely coincides with the acquisition of maturation-dependent alcohol resistance.

The NO-cGMP-PKG pathway utilizes NF- κ B to signal its neuroprotective effects

The precise mechanism by which the NO-cGMP-PKG pathway protects neurons against alcohol toxicity is not known. However, the pathway's third step, activation of PKG, is critical for its neuroprotective effect (Bonthius et al., 2004a; Hutton-Kahrberg et al., 2004). Thus, we hypothesized that a neuroprotective protein whose activity depends on PKG-mediated phosphorylation is the downstream effector molecule of the pathway. One candidate is the transcription factor NF- κ B. NF- κ B is a neuroprotective molecule that can protect developing neurons against cell-death from certain insults, such as low potassium (Chin et al., 2004). Activation of NF- κ B requires phosphorylation of IkappaB, which leads to its dissociation from NF- κ B. This results in the translocation of NF- κ B to the nucleus, where it binds to the regulatory element of target genes and activates their transcription (Gilmore, 2006). Phosphorylation of NF- κ B is critical for its survival promoting effects in neurons (Liu and D'Mello, 2006). Although IkappaB is phosphorylated most commonly by IkappaB kinase (IKK), PKG can also specifically phosphorylate IkappaB (He and Weber, 2003). Furthermore, cGMP drives PKG-induced phosphorylation of IkappaB (He and Weber, 2003). We reasoned that, if NF- κ B is downstream of the NO-cGMP-PKG pathway, then inhibition of NF- κ B should abolish the neuroprotective effect of the pathway against alcohol toxicity, even if the pathway is stimulated upstream (Bonthius et al., 2009).

Through a series of "block and rescue" experiments, we found that the NO-cGMP-PKG pathway does, indeed, signal its neuroprotective effects against alcohol via NF- κ B (Figure 1). In particular, we found that alcohol alone killed 14.2% of cultured cerebellar granule cells, but the cell death increased to 24% when a specific NF- κ B inhibitor (3-chloro-4-nitro-N-(5-nitro-2-thiazolyl)-benzamide) was added to the cultures. When the cultures were treated with an upstream activator of the pathway (the NO generator, DETA-NONOate) together with NF- κ B inhibitor, the upstream activator's protective effect observed when used alone, was abolished (Bonthius et al., 2009). On the other hand, activation of NF- κ B

with TNF- α protected the cells, even if the NO-cGMP-PKG pathway was inhibited upstream with L-NAME or LY-83583. In addition, NF- κ B inhibitor had no effect on alcohol-induced cell death in cultures lacking nNOS (Bonthius et al., 2008). Alcohol-induced cell death was higher in nNOS $^{-/-}$ cultures than in wild type cultures, and the percent cell death remained unchanged in the presence of NF- κ B inhibitor (Bonthius et al., 2008), indicating that NF- κ B must interact with a signaling cascade involving NO. These results demonstrate that the neuroprotective effect of the NO-cGMP-PKG pathway is mediated by NF- κ B and that nNOS depends upon NF- κ B to exert its neuroprotective activity.

How NF- κ B protects developing neurons against alcohol toxicity is not known. However, because NF- κ B is a transcription factor, it may protect neurons by increasing the expression of neuroprotective genes. However, the identities of these genes are yet to be determined.

Lack of nNOS function exacerbates alcohol's neuroteratogenic effects in vivo.

After discovering that neurons genetically deficient for nNOS (nNOS $^{-/-}$) have an increased vulnerability to alcohol-induced cell death *in vitro*, we next examined whether these cells also have an increased vulnerability *in vivo*. To examine this possibility, we compared the teratogenicity of alcohol in wild type and nNOS $^{-/-}$ mouse strains. It is noteworthy to mention that, although nNOS $^{-/-}$ mice manifest some alterations in gastrointestinal structure and function, the gross and fine morphologies of their brains are normal. In addition, we have demonstrated that nNOS $^{-/-}$ mice generate and maintain normal complements of CNS neurons (Bonthius et al., 2004b). Thus, differential effects of agents on nNOS $^{-/-}$ and wild type mice can be detected and measured with relative ease.

We exposed neonatal mice from both strains to acute doses of alcohol (0.0, 2.2, 3.3, or 4.4 mg/g) daily from postnatal day (PD) 4 through 9. This pattern of alcohol exposure, consisting of a single alcohol dose, mimics the "binge" drinking that is commonly observed among women who abuse alcohol during pregnancy (Anderson et al., 2014). We chose this timing of alcohol administration (PD 4-9) because, in terms of brain development, the first two weeks of rodent life correspond to the third trimester of human gestation (Dobbing and Sands, 1979; Goodlett and Johnson, 1999). This period of development encompasses the "brain growth spurt," which is a period of rapid brain growth during which the developing brain is particularly vulnerable to teratogens, including alcohol (Dobbing, 1981).

Following the alcohol exposures, we quantified the surviving neurons in a variety of brain regions, including the cerebellum, hippocampus, dentate gyrus, and cerebral cortex (Bonthius et al., 2002). We found that alcohol reduced neuronal survival in a dose-related fashion in both genotypes and in all brain regions. However, the magnitude of neuronal losses was more severe in the nNOS $^{-/-}$ mice than in wild type in all of the brain regions examined. Furthermore, while the lowest dose of alcohol (2.2 mg/g/day) did not affect neuronal survival in the wild type mice, this same dose did significantly reduce neuronal survival in several brain regions of the nNOS $^{-/-}$ mice. Thus, lack of a functional nNOS gene not only increases the severity of alcohol-induced neuronal losses, but also reduces the

threshold dose of alcohol necessary to cause neuronal death. These results demonstrate that nNOS plays a key role in protecting neurons against alcohol toxicity *in vivo* and that null mutation of this gene substantially worsens alcohol-induced neuronal losses.

Mutation of nNOS makes the developing brain vulnerable to a single dose of alcohol.

In most cases of documented FAS, the human fetus has been exposed to large quantities of alcohol on multiple occasions during gestation. However, it is possible that a genetically susceptible fetus could be damaged by a single exposure to alcohol - an exposure that might have no evident impact on genetically less susceptible individuals. This is an important issue because, while most women reduce their alcohol intake during pregnancy, many continue to drink intermittently, believing, sometimes upon the advice of their obstetricians, that low levels of alcohol consumption during pregnancy are harmless (Kitsantas et al., 2014; Watt et al., 2014).

To answer this question we treated nNOS^{-/-} and wild type mice with a single dose of alcohol (4.4 mg/g) on a single occasion (PD 4) and quantified surviving neurons in the cerebral cortex and several regions of the hippocampal formation (de Licona et al., 2009). We found that the single alcohol exposure caused substantial neuronal losses in nNOS^{-/-} mice, while causing no neuronal losses in wild type mice. These results demonstrate that, during fetal development, a single exposure to alcohol that has no evident effect on most fetuses can damage the brains of genetically vulnerable individuals. This is a clinically important principle because it strongly suggests that women should completely avoid alcohol during pregnancy, since they might be carrying a fetus that is genetically susceptible to damage, even from relatively small alcohol quantities that would be innocuous to most. This recommendation that all pregnant women should avoid alcohol should remain in place until whole genome mapping becomes routine and until the genes that influence fetal vulnerability to alcohol are all identified.

The greater neuronal losses following alcohol exposure in nNOS^{-/-} mice than in wild type mice is not due to differences in alcohol metabolism between the two strains. We have shown that, following any given alcohol dose, wild type and nNOS^{-/-} mice have virtually identical peak blood alcohol concentrations and rates of alcohol clearance (Bonthius et al., 2002). Furthermore, the fact that isolated cerebellar granule cells in culture exhibit greater vulnerability when they are derived from nNOS^{-/-} mice than from wild type mice strongly suggests that the increased vulnerability is due to a factor intrinsic to the neurons themselves and not due to dysfunction of another organ system or external factor (Bonthius et al., 2002; Hutton-Kahrberg et al., 2004).

Lack of nNOS worsens the behavioral deficits induced by developmental alcohol exposure.

People with FASD exhibit numerous behavioral problems, including hyperactivity, attention deficits, impaired coordination, poor memory, poor reasoning and judgment, and learning disabilities (Riley et al., 2011). The severity of these behavioral deficits varies considerably

among individuals, and this variance is likely due, at least in part, to genetic differences. Although a limited number of genes have been identified that alter the vulnerability of the developing brain to alcohol (Bonthius et al., 2002; Chen et al., 2011; Heaton et al., 2006; Noel et al., 2011; Young et al., 2003), it is not known whether these differential neuronal vulnerabilities translate into altered behavioral deficits later in life. For this reason, we conducted experiments to determine whether lack of a functional nNOS gene can worsen alcohol-induced learning, attention, and behavioral deficits in adulthood. To test our hypothesis, we treated wild type and nNOS^{-/-} mice with alcohol (0.0 to 4.4 g/kg) daily over postnatal days 4 through 9. As demonstrated above, this results in acute neuronal losses that are significantly more severe in nNOS^{-/-} mice than in wild type (Bonthius et al., 2002). We then allowed these mice to grow to adulthood.

Beginning on PD 85, we subjected the mice to a series of behavioral tests, including open field activity, Morris water maze, and prepulse inhibition. These tests detect abnormalities in activity levels, memory, and sensori-motor gating, respectively, all of which are abnormal in many people with FASD. Furthermore, all of these tests rely on function of the forebrain, a major target of alcohol in the developing brain.

We found that the alcohol exposure early in life impaired performance on all three of these tests during adulthood (Karacay et al., 2014a). However, the extent of the behavioral impairment was much worse in the nNOS^{-/-} mice than in the control mice. In fact, on several of the tests, alcohol exposure had only a minimal effect on the wild type mice, but substantially impaired performance in the nNOS^{-/-} mice. Furthermore, the cell count studies showed that alcohol-induced reductions in cell number were significantly correlated with performance deficits in the nNOS^{-/-} mice, but not in the wild type mice (Karacay et al., 2014b). These results demonstrated 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. The results also mimic 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 and Hannigan, 1995; Streissguth et al., 1994). The findings provide evidence that these qualitative outcome differences are likely due, in large part, to genetic differences.

Summary and Conclusions

Despite warnings from private and governmental organizations discouraging alcohol use during pregnancy, many pregnant women continue to drink. This is partly because of a belief that intermittent alcohol consumption would not harm a fetus. However, due to genetic differences, some fetuses are much more vulnerable to alcohol-induced brain injury than others. Our findings regarding the nNOS gene and its role in neuroprotection against alcohol toxicity described in this review clearly demonstrate that the genetic composition of an individual is of paramount importance in determining his or her vulnerability to alcohol toxicity.

We have found that the product of nNOS, nitric oxide, acts in a variety of ways to protect the developing brain against alcohol toxicity. By activating the NO-cGMP-PKG pathway, nitric oxide protects neurons against alcohol-induced cell death. In addition, it plays a key role in maturation-dependent alcohol resistance, which allows maturing neurons to escape alcohol's damaging effects, and it is instrumental in allowing several growth factors, including FGF-2 and NGF, to exert their neuroprotective effects.

Using a mouse model, we have found that targeted disruption of the nNOS gene substantially worsens alcohol-induced neuronal losses and behavioral deficits. In nNOS^{-/-} mice, alcohol induces histologic changes and cognitive deficits that are not seen in wild type mice. This is similar to the human condition, in which alcohol causes distinct and substantial problems in certain individuals, while it leaves others apparently unaffected.

Whether variations in nNOS and its expression play a role in the severity of FASD in humans is unknown. Complete genetic deficiency of nNOS in humans has not been described to date. However, polymorphisms in the nNOS gene have been reported in different ethnic populations (Grasemann et al., 1999) and have been shown to influence susceptibility to several diseases, including infantile pyloric stenosis, Parkinson's disease, epilepsy, and major depression (Chung et al., 1996; Galecki et al., 2011; Levecque et al., 2003; M.A.Sayitoglu et al., 2006; Sarginson et al., 2014). Certain nNOS polymorphisms may similarly underlie or contribute to increased alcohol vulnerability in humans.

nNOS is the only gene discovered to date whose mutation *worsens* alcohol-induced brain damage. However, several other genes have been discovered whose mutation can *protect* the developing brain against alcohol toxicity. These genes include tPA and BAX, both of which play a role in triggering apoptosis (Heaton et al., 2006; Noel et al., 2011; Young et al., 2003). Mutation of these genes prevents alcohol-induced apoptosis, and thus preserves the lives of neurons that would otherwise have been killed by alcohol. Thus, the effect of individual genes on the alcohol-exposed developing brain can range from strongly positive to strongly negative, and the net genetic influence is likely a complex interplay of genes that worsen and ameliorate alcohol's teratogenic effects

Can our finding that NO protects the developing brain against alcohol toxicity be useful clinically? It is not yet known whether *in vivo* stimulation of the NO-cGMP-PKG pathway can protect developing neurons against alcohol toxicity. Transgenic mice overexpressing nNOS in vulnerable neuronal populations or pharmacological stimulation of the pathway *in vivo* could answer this question. In fact, our current studies show that pharmacological stimulation of the NO-cGMP-PKG pathway in mice can reduce expression of cleaved caspase-3, an apoptotic marker that acutely rises in the developing cerebral cortex following alcohol exposure (unpublished data). Thus, the nNOS gene and the NO-cGMP-PKG pathway may have substantial clinical relevance in protecting the developing brain against alcohol toxicity.

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Figure 1.

The NO-cGMP-PKG pathway, along with its inhibitors, activators, and downstream effectors. Nitric oxide (NO) is produced within neurons through the catalytic actions of neuronal nitric oxide synthase (nNOS). Nitric oxide activates soluble guanylyl cyclase (sGC), which synthesizes cyclic GMP (cGMP). This second messenger activates cGMP-dependent protein kinases (PKG). These protein kinases exert their effects by phosphorylating target proteins, one of which is the transcription factor, NF-κB. This transcription factor alters gene expression in ways that protect neurons against alcohol-induced cell death. Pathway activators are depicted by open arrows, while pathway inhibitors are depicted by vertical bars. NMDA promotes the pathway by raising intracellular calcium levels, which activates nNOS. DETA-NONOate promotes the pathway by serving as an NO generator. Bromo-cyclic GMP (8-Br-cGMP) is a cyclic GMP analogue that activates PKG. Tumor necrosis factor alpha (TNF-α) is a cytokine that promotes the pathway by activating NF-κB. NAME (N^G-Nitro-L-arginine-methyl ester) inhibits the pathway by blocking NO production by nNOS. LY-83583 inhibits the pathway by blocking cGMP production by sGC. PKG inhibitor (PKGi) blocks the pathway by inhibiting the kinase activity of PKG. NF-κB inhibitor (NFi) inhibits the pathway by blocking NF-κB activation. We utilized a series of “block and rescue” experiments to demonstrate that the NO-cGMP-PKG pathway protects developing neurons against alcohol toxicity and that it produces this protective effect through the actions of NF-κB.

Table 1

Neuronal survival-promoting effects of intrinsic growth factors on cerebellar granule cell cultures and reliance of the growth factors on the NO-cGMP-PKG pathway to produce their effects.

Growth factor	Neurotrophic Effect or Neuroprotective Effect Against Alcohol?	Utilizes the NO-cGMP-PKG Pathway for survival-promoting effect?
Basic fibroblast growth factor (FGF-2)	Both	Yes
Nerve growth factor (NGF)	Neuroprotective	Yes
Insulin-like growth factor (IGF-1)	Neurotrophic	Yes
Brain-derived neurotrophic factor (BDNF)	Both	No
Epidermal growth factor (EGF)	Neither	NA