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The nitric oxide signaling pathway inhibits intracellular calcium release to prevent neurodevelopmental alcohol toxicity

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University of Iowa

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THE NITRIC OXIDE SIGNALING PATHWAY INHIBITS INTRACELLULAR
CALCIUM RELEASE TO PREVENT NEURODEVELOPMENTAL ALCOHOL
TOXICITY

by

Dimitrios Elias Kouzoukas

An Abstract

Of a thesis submitted in partial fulfillment of the
requirements for the Interdisciplinary Studies-
Ph.D. degree in Neurobiology
in the Graduate College of
The University of Iowa

December 2010

Thesis Supervisor: Professor Nicholas J. Pantazis

ABSTRACT

In the context of fetal alcohol spectrum disorders, we investigated how the nitric oxide (NO) signaling pathway influences intracellular calcium (Ca^{2+}) to mediate alcohol resistance, using a primary cell culture model of cerebellar granule neurons (CGN). Alcohol during fetal brain development triggers abnormally high apoptotic cell death in vulnerable neuronal populations, culminating in serious behavioral and cognitive deficits that persist into adulthood. Prior studies demonstrated that the NO signaling pathway [neuronal nitric oxide synthase \rightarrow NO \rightarrow soluble guanylyl cyclase \rightarrow cyclic guanosine monophosphate \rightarrow protein kinase G (PKG)] mitigates alcohol toxicity, consequently diminishing neuronal loss both *in vivo* and *in vitro*. Endoplasmic reticulum (ER) Ca^{2+} release, a key apoptotic mechanism, requires the inositol 1,4,5-trisphosphate receptor (IP_3R), a known PKG substrate. Our studies focused on this crucial intersection point where the NO signaling cascade can influence Ca^{2+} -mediated apoptotic mechanisms, and exposed a downstream mechanism where NO can moderate alcohol neurotoxicity.

We hypothesized that as alcohol disturbs neuronal Ca^{2+} homeostasis to trigger cell death, the NO signaling pathway counters it by limiting Ca^{2+} release from the ER. We examined first the role of the phospholipase C (PLC) pathway [PLC \rightarrow inositol 1,4,5-trisphosphate \rightarrow IP_3R \rightarrow Ca^{2+}] in developmental neurotoxicity through our *in vitro* CGN model, extending previous *in vivo* studies. We found that alcohol terminates developing neurons by eliciting abnormal Ca^{2+} release from the ER rather than from an extracellular source, via a PLC – IP_3R -dependent signaling mechanism. Inhibiting either calcineurin or Ca^{2+} / calmodulin-dependent protein kinase ii (CaMKii), which participate in parallel Ca^{2+} -activated apoptotic cascades, shielded CGN cultures from alcohol. Blocking the mitochondrial Ca^{2+} uniporter or the mitochondrial permeability transition pore also provided neuroprotection. That the activated pathways must interact to generate cell

death likely explains why inhibiting one of multiple parallel signaling cascades limits alcohol toxicity.

We next demonstrated that activating the NO pathway downstream at PKG eliminated both alcohol-related neuronal death and the accompanying rapid rise in intracellular Ca^{2+} , an effect that markedly resembled IP_3R inhibition. Experiments that temporally manipulated the addition of PKG activators in relation to alcohol exposure linked PKG's obstruction of alcohol-induced Ca^{2+} elevations to alcohol resistance. In contrast, brain-derived neurotrophic factor (BDNF), which does not rely on PKG to provide neuroprotection, failed to block alcohol-induced Ca^{2+} elevations while preventing alcohol toxicity. This indicates that although PKG blocks alcohol-induced Ca^{2+} elevations, averting these Ca^{2+} elevations is not necessary for neuroprotection. BDNF may confer alcohol resistance through an as yet unidentified process downstream from the disruption of intracellular Ca^{2+} .

In summary, we established that 1) alcohol induces toxic Ca^{2+} elevations originating from the ER through a PLC – IP_3R -dependent pathway, and that 2) PKG-mediated alcohol resistance is linked to preventing the intracellular Ca^{2+} surges. These findings support the hypothesis that the NO signaling pathway shields developing neurons from alcohol by limiting Ca^{2+} release from the ER.

Abstract Approved: _____

Thesis Supervisor

Title and Department

Date

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Graduate College
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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee
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To Victoria

“Come, Watson, come! The game is afoot. Not a word! Into your clothes and come!”
Sherlock Holmes

Sir Arthur Conan Doyle, *The Adventure of the Abbey Grange*

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LIST OF ABBREVIATIONS

2-APB	2-aminoethoxydiphenyl borate
5-HT ₃	5-hydroxytryptamine type 3
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
BAC	blood alcohol content
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologous antagonist/killer
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BAPTA/AM	BAPTA acetoxymethyl ester
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-X _L	BCL-2 extra large
BDNF	brain-derived neurotrophic factor
Ca ²⁺	ionic calcium or calcium
[Ca ²⁺] _i	concentration of intracellular calcium
CaMKii	calcium/calmodulin-dependent protein kinase ii
cGMP	cyclic guanosine monophosphate
CGN	cerebellar granule neuron
CICR	calcium-induced calcium release
CNS	central nervous system
CO ₂	carbon dioxide

DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDRF	endothelium-derived relaxing factor
EGTA	ethylene glycol tetraacetic acid
EGTA/AM	EGTA acetoxymethyl ester
eNOS	endothelial NOS
ER	endoplasmic reticulum
EtOH	ethanol
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
GABA	γ -aminobutyric acid
GDP	guanosine diphosphate
GIRK	G-protein coupled inward rectifying potassium channels
GSK-3 β	glycogen synthase kinase-3 beta
GTP	guanosine triphosphate
HEBSS	HEPES-buffered Earle's balanced salt solution
IP ₃	inositol trisphosphate
IP ₃ R	inositol trisphosphate receptor
IP ₃ R1	IP ₃ R, type I
IP ₃ R2	IP ₃ R, type II
IP ₃ R3	IP ₃ R, type III
iNOS	inducible NOS
IRAG	IP ₃ R-associated cGMP kinase substrate
LTD	long-term depression
MCU	mitochondrial calcium uniporter

MEOS	microsomal ethanol-oxidizing system
mHCX	mitochondrial H ⁺ /Ca ²⁺ exchanger
mNCX	mitochondrial Na ⁺ /Ca ²⁺ exchanger
MPTP	mitochondrial permeability transition pore
MRI	magnetic resonance imaging
Na ⁺	sodium ion
NAD ⁺	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCX	Na ⁺ / Ca ²⁺ exchanger
NMDA	N-methyl-d-aspartate
nNOS	neuronal NOS
nNOS ^{-/-}	nNOS knockout
NO	nitric oxide
NOS	nitric oxide synthase
NT-3	neurotrophin 3
PBS	phosphate buffered saline
PDL	poly-D-lysine
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PKG-I α	PKG, type I α
PKG-I β	PKG, type I β
PKG-II	PKG, type II
PLC	phospholipase C
PMCA	plasma membrane Ca ²⁺ -ATPase
PTI	Photon Technologies Incorporated
RyR	ryanodine receptor

SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
sGC	soluble guanylyl cyclase
UV	ultraviolet
VDAC	voltage-dependent anion channel
X-C	xestospongin C

CHAPTER I

INTRODUCTION

Fetal Alcohol Syndrome Disorders

History

In 1973, Jones and Smith coined the term Fetal Alcohol Syndrome (FAS) to describe a pattern of altered morphogenesis and characteristics resulting from prenatal alcohol exposure (Jones & Smith 1973). Identified were a distinctive pattern of pre- and postnatal growth deficiencies, craniofacial abnormalities and defects in major organ systems in eight unrelated children born to alcoholic mothers. Naming the syndrome in 1973 drew attention to alcohol abuse as a serious public health issue, encouraging research into the basic mechanisms of prenatal alcohol toxicity and its long-term consequences. Subsequent research and clinical experience suggested that prenatal alcohol exposure leads to a broad spectrum of teratogenic deficits extending far beyond the original case definition. The term Fetal Alcohol Spectrum Disorder (FASD) was developed to include FAS as well as other deleterious outcomes that result from heavy prenatal alcohol exposure (Greenbaum & Koren 2002).

Epidemiology

FAS is the single leading preventable cause of mental retardation in the United States with reported cases ranging from 0.2 to 2 cases per 1000 births (Riley & McGee 2005), although FASD has been conservatively reported as being 1 in 100 births (Sampson *et al.* 1997). Sadly, despite widespread knowledge of alcohol's devastating teratogenicity, a study of women of childbearing age (18 to 44 years) revealed that 10% drank alcohol while pregnant and 2% engaged in binge drinking (Sidhu & Floyd 2002). Influencing risk factors for FAS births are race (Abel & Sokol 1987), socioeconomic status (Sokol *et al.* 2003), and the local prevalence of binge drinking (May & Gossage

2001a), with increased risk among African Americans (Abel & Sokol 1987), Native Americans (May & Gossage 2001b), and those with low socioeconomic status (May & Gossage 2001a). Additional maternal risk factors include an age of over 30 years and a history of binge drinking (Sokol et al. 2003).

Clinical Manifestations

The diagnostic criteria recommended by the Center for Disease Control (Bertrand *et al.* 2005) mandate that FAS diagnoses require evidence of the following: 1) dysmorphic facial features consisting of a smooth philtrum, a thin vermilion, and small palpebral fissures; 2) growth retardation (deficient prenatal or postnatal growth); and 3) central nervous system (CNS) abnormalities which include structural, neurological, and functional defects that often manifest as microencephaly, epilepsy, and severe behavioral and cognitive impairments. Although once a requirement, current guidelines no longer entail verifying maternal alcohol use during pregnancy due to the difficulty of obtaining accurate reports (Riley & McGee 2005). Other alcohol-related birth defects not incorporated into the FAS diagnosis commonly include cardiac ventricular septal defects (Steege & Woolf 1979), and urogenital anomalies such as renal hypoplasia or agenesis (Abel 1984).

Although alcohol-related growth and facial abnormalities gradually diminish as affected children mature, the effects of CNS damage persist and are therefore considered the most debilitating. That children with and without dysmorphic features share neurocognitive deficits to a similar degree (Mattson *et al.* 1997) highlights the sensitivity of the developing CNS to alcohol exposure. Studies examining the neuropsychological profile of individuals with FASD have found performance deficits with increased task complexity across multiple functional domains including that of language, visual perception, memory and learning, social functioning, and number processing. This is consistent with the conclusion that children with FASD have a global difficulty with

information processing and integration (Kodituwakku 2009), which may be presumed to stem from altered brain function and structure (Roebuck *et al.* 1998).

Fetal Alcohol Effects in the Brain

Alcohol Neuropharmacology

Alcohol (ethanol, ethyl alcohol) is a small water-soluble molecule, which after ingestion is rapidly absorbed and completely distributed to all body tissues. Since women have relatively lower body water content than men, women attain higher peak blood alcohol content (BAC) levels from an equivalent oral alcohol dose than men and therefore are more susceptible to alcohol effects. Alcohol concentrations in cerebral tissues rapidly climb given that the brain receives 15 - 20% of the cardiac output and that alcohol readily crosses biological membranes.

Over 90% of ingested alcohol is metabolized by alcohol dehydrogenase, a cytosolic NAD-dependent enzyme, and by the microsomal ethanol-oxidizing system (MEOS) to metabolize alcohol into acetaldehyde (Masters & Lee 1998). When BAC levels are lower than 100 mg/dl, alcohol dehydrogenase, located in the liver and gastrointestinal track, consumes nicotinamide adenine dinucleotide (NAD⁺) to biotransform alcohol (Lieber & DeCarli 1968). Since the fetal liver shows very little alcohol dehydrogenase activity, alcohol clearance primarily depends on the maternal liver (Clarke *et al.* 1989). At BAC levels above 100 mg/dl, the MEOS contributes more to alcohol metabolism, as alcohol dehydrogenase depletes NAD⁺ levels (Teschke *et al.* 1974). In normal individuals, a BAC ranging from 50 mg /dl to 80 mg/dl alcohol usually results in impaired driving ability; from 120 to 160 mg/dl, gross drunkenness; greater than 300 mg/dl, stupor anesthesia and coma; and above 500 mg/dl, respiratory depression and death (Becker 1984). MEOS efficiency increases with habitual alcohol abuse and thus is responsible for building alcohol tolerance, so that a BAC of 300 - 400 mg/dl in a

chronic drunk may only result in the appearance of slight intoxication or even sobriety (Lieber & DeCarli 1968, Lieber 1999).

It is important to note that alcohol's ability to dissolve into lipid membranes (Deitrich *et al.* 1989) enables it to affect a vast array of bimolecular processes given a great enough concentration. Nevertheless, even at the physiological concentrations resulting from recreational alcohol use, alcohol affects neurotransmitter receptors for γ -aminobutyric acid (GABA), glycine, glutamate, opioids, serotonin, and acetylcholine; potassium and calcium ion (Ca^{2+}) channels; enzymes such as phospholipase C (PLC), adenylyl cyclase, and cytochrome P-450; the sodium-potassium pump; and electron transport (Mattila 1990, Deitrich *et al.* 1989). Considerable attention has been given to alcohol's abilities to augment GABA-mediated synaptic inhibition (Ticku & Kulkarni 1988) and to suppress N-methyl-d-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor activity (Weight *et al.* 1991). Alcohol also potentiates 5-hydroxytryptamine type III receptors (5-HT_3), which have been implicated in nausea and vomiting (Lovinger & White 1991); and G-protein coupled inward rectifying potassium channels (GIRKs), which have been implicated in alcohol's analgesic effects (Kobayashi *et al.* 1999).

This large repertoire of pharmacodynamic interactions discounts the possibility that a single site can be responsible for alcohol-induced birth defects. Even so, specific mechanisms contributing to alcohol's teratogenicity have been identified and include interference with neurotrophins, intracellular Ca^{2+} signaling, and cholinergic neurotransmission.

Altered Brain Morphology

As mentioned, microencephaly is a prevalent feature in FAS. Autopsy studies often described extensive cerebral dysgenesis and neuronal migration defects (Roebuck *et al.* 1998). In children with severe FAS, underdeveloped cerebral hemispheres and fused

anterior frontal lobes (Coulter *et al.* 1993), a condition reminiscent of mild holoprosencephaly (Sulik & Johnston 1982), have been reported. Additional malformations include agenesis of the corpus callosum and anterior commissure; absent olfactory bulbs; and malformed hippocampi, basal ganglia, and brainstem (Peiffer *et al.* 1979). Autopsy studies have also found severe errors in neuronal migration resulting in cortical and cerebellar disorganization (Clarren *et al.* 1978) and a reduction of the number of dendritic spines (Ferrer & Galofre 1987). The cerebellum shows severe vulnerability to prenatal alcohol exposure in that cases of cerebellar hypoplasia and dysgenesis are consistently seen (Roebuck *et al.* 1998).

Structural brain imaging studies using magnetic resonance imaging (MRI) support autopsy findings revealing a decline in overall brain size and volumetric reductions in each cerebral cortical lobe, the corpus callosum, basal ganglia, hippocampus, and cerebellum (Norman *et al.* 2009). Volumetric analyses allow correction for the alcohol-induced reduction in overall brain size in order to detect regional brain sensitivities. These studies still demonstrated relative volumetric reductions in the parietal lobe (Archibald *et al.* 2001), the corpus callosum (Sowell *et al.* 2001), the caudate nucleus (Mattson *et al.* 1996), and in the cerebellum (Sowell *et al.* 1996, O'Hare *et al.* 2005, Archibald *et al.* 2001). Within the cerebellum, the greatest relative reductions were observed in the anterior vermis (O'Hare *et al.* 2005, Sowell *et al.* 1996). Taken together, these findings suggest that the susceptibility to prenatal alcohol exposure differs among brain regions.

Rodent neurodevelopmental studies typify findings in humans reliably demonstrating the dependence of microencephaly severity on the extent of the prenatal alcohol exposure (Samson & Diaz 1981, Samson *et al.* 1982, Samson & Grant 1984, West *et al.* 1984). Similar to that seen human autopsy studies, alcohol induces cortical (Miller 1986, Miller 1993) and cerebellar (Kumada *et al.* 2007) migrational defects in rodents. Dramatic cell losses have also been observed in specific rodent brain regions

including the cerebellum (Goodlett *et al.* 1990, Maier *et al.* 1997, Bonthius *et al.* 2002, Chen *et al.* 1998), the hippocampus (Livy *et al.* 2003), and the olfactory bulbs (Maier *et al.* 1999, Chen *et al.* 1999b).

Despite the seeming widespread teratogenic effects of alcohol exposure, there are resistant brain regions. Gestational and neonatal alcohol exposure of rats did not reduce cell numbers in the ventrolateral nucleus of the thalamus (Livy *et al.* 2001) or in the locus coeruleus (Chen *et al.* 1999a).

Cerebellar Damage

Both human and animal studies have demonstrated that the developing cerebellum is one of the most susceptible brain regions to alcohol exposure (Roebuck *et al.* 1998, Pierce *et al.* 1999), damage to which is associated with severe deficits in motor skills and learning (Riley & McGee 2005, Niccols 2007). In both mice and rats, alcohol exposure during gestation or the postnatal period reduces cerebellar size (Nathaniel *et al.* 1986, Bonthius *et al.* 2002), severely depleting granule and Purkinje neurons of the cerebellar cortex (Hauser *et al.* 2003, Pierce *et al.* 1999, Chen *et al.* 1998, Borges & Lewis 1983a, Bonthius & West 1990). That cell loss appears greatest in cerebellar lobules with Purkinje cells in the process of extending dendrites suggests that alcohol's teratogenicity may stem from an effect on neuronal maturation (West *et al.* 1990).

Significant correlation exists between Purkinje and granule cell loss, which may indicate that the decline in cerebellar granule neurons (CGN) results from the loss of their Purkinje cell targets (Bauer-Moffett & Altman 1977, Borges & Lewis 1983b). However, evidence exists to suggest that alcohol affects CGNs directly. During development of the cerebellar cortex, CGNs normally migrate from the external granule layer through the molecular layer to the internal granule layer (Jiang *et al.* 2008). Alcohol delays CGN migration to their final destination in a dose-dependent manner (Kumada *et al.* 2006). Consistent with this finding, extensive migrational defects are often observed, such as

solitary or ectopic granule cells in the molecular layers of alcohol exposed rats (Sakata-Haga *et al.* 2001). Alcohol also affects CGN viability. In CGN cultures derived from neonatal mice, alcohol addition results in an ethanol dose-dependent cell death (Pantazis *et al.* 1993).

Prenatal alcohol exposure also affects how remaining Purkinje and CGNs function. Recordings of spontaneous Purkinje cell activity revealed decreased complex spiking activity (Backman *et al.* 1998), which is indicative of abnormal climbing fiber input from the inferior olive (Napper & West 1995). Linked to the loss of Purkinje cell function in rodents is poor motor skills performance on rotating rod (Goodlett *et al.* 1991, Klintsova *et al.* 1998) and parallel bar tests (Meyer *et al.* 1990, Thomas *et al.* 1996), as well as on the acquisition of classical eyeblink conditioning (Green 2004). However, tests of eyeblink conditioning are functional tests of an entire neural circuit and therefore also reflect additional alcohol-induced cell losses in the deep cerebellar nuclei (Green *et al.* 2002), especially of the interpositus nucleus and in the inferior olive of the brainstem (Green 2004).

Neonatal alcohol exposure also affects the development of cerebellar neurotransmitter systems. Inhibition of NMDA receptor-mediated Ca^{2+} and electrophysiological responses have been consistently demonstrated both *in vitro* (Wegelius & Korpi 1995, Groul 1992, Gruol & Parsons 1996, Hoffman *et al.* 1989b) and *in vivo* (Simson *et al.* 1991, Popp *et al.* 2008, Thomas *et al.* 2001). It also has been shown that alcohol diminishes muscarinic receptor-stimulated phosphoinositide metabolism in cerebellar slices from 7 day old rats (Balduini & Costa 1990, Balduini *et al.* 1991). Downstream from muscarinic receptors, ethanol reduces inositol trisphosphate receptor (IP₃R) binding in the cerebellum (Nio *et al.* 1991). Detrimental alcohol-induced alterations in neurotrophin systems that are important for neuronal maturation and survival are observed as well. Alcohol reduces levels of neurotrophin receptors, TrkA, TrkC, and of p75 (Moore *et al.* 2004, Dohrman *et al.* 1997) on neonatal Purkinje cells,

and decreases brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) secretion in CGN cultures (Heaton *et al.* 2004). Alcohol-induced reductions of BDNF and NT-3 levels are known to affect CGN motility and contribute to the severe migrational defects observed in FAS (Jiang *et al.* 2008).

Temporal Windows of Vulnerability during Fetal

Development

In a review of maternal alcohol consumption during pregnancy, women who reported drinking identical quantities of alcohol delivered babies with considerable variability in the FAS symptoms (Abel & Sokol 1986). One explanation for this phenomenon is that there are “critical” periods during brain development at which the brain is especially vulnerable to alcohol, making the timing of alcohol exposure a key factor in the severity of the resulting brain damage (West 1987). Presumably pregnant women that drank during “critical” periods would birth children with more severe FAS symptoms than those that drank during non-vulnerable periods.

Indeed studies in rodent neurodevelopment models show temporal windows of alcohol vulnerability during brain development (West *et al.* 1994). Alcohol exposure during the first half of gestation in rats (equivalent to the first trimester in humans) produced face (Sulik *et al.* 1981) and limb dysmorphology (Webster *et al.* 1983), along with neural tube closure defects (Wynter *et al.* 1983). These effects are similar to those associated with FAS in humans. Pregnant Long-Evans rats fed ethanol also showed neuronal depletion in pups equivalent to that seen in humans (Miller & Potempa 1990). At this period of development, proliferation of neuronal precursors occurs along with organogenesis and formation of the neural tube and crest (Kandel *et al.* 2000). Although alcohol exposure during later developmental periods also induces severe neuronal loss, the likelihood of craniofacial anomalies diminishes (Sulik & Johnston 1983, Phillips & Cragg 1982, Ernhart *et al.* 1987).

The second critical period of alcohol susceptibility in rats occurs from gestational days 11 to 21 (the second trimester rat equivalent of human gestation), a time in which most brain areas except the cerebellum are differentiating and when neuronal proliferation and migration occurs (Guerra 1998). Postmitotic neurons leaving from germinal zones migrate using important directional cues from radial glia (Rakic 1991). Alcohol exposure at this stage accelerates radial glial transformation into astrocytes in the cortex, thus depriving late-generated migrating neurons of their radial glial guides (Miller & Robertson 1993). These ethanol-induced changes in glia likely underlie the abnormal neural migration defects detected in FAS. Alcohol exposure also diminishes the generation of hippocampal neurons (Miller 1995) and significantly reduces neuronal densities in the ventromedial hypothalamus, and in the mediodorsal and ventral thalamus (Ikonomidou *et al.* 2000).

In rodents, the first two weeks following birth mirror the third trimester of human pregnancy, a time in which rapid brain growth and maturity transpires (Dobbing & Sands 1979). This period of greater vulnerability coincides with synaptogenesis, which in humans extends from the sixth month of pregnancy to years after birth (Dobbing & Sands 1973, Dobbing & Sands 1979). This brain development period is especially sensitive to alcohol exposure, being when a major increase in brain weight occurs along with glial proliferation and maturation, synaptogenesis, and dendritic arborization. At the same time, the cerebellum also undergoes its most rapid developmental growth spurt (Dobbing & Sands 1973).

The cerebellum is especially vulnerable at this time. Administration of ethanol to neonatal rats on postnatal days 4 through 7 but not on postnatal days 8 through 12 resulted in extensive depletion of cerebellar granule and Purkinje neurons (Hamre and West, 1993). This finding is in agreement with a later study that examined alcohol-induced cell loss in pups exposed on postnatal days 4 through 9 (Maier *et al.* 1999). However extensive alcohol-induced neuronal loss has been observed in numerous other

rat brain regions, including the olfactory lobes, frontal and parietal cortex, the caudate, and hippocampus, all of which are sensitive to alcohol from the last days of rat embryonic development to the first two weeks after birth (Maier et al. 1999, Ikonomidou et al. 2000). A comparison of brain region to body weight ratios in 10 day old pups found the greatest growth deficits in brain, forebrain, and cerebellum among pups that received alcohol during the third trimester human equivalent (Maier et al. 1997). Alcohol's effect on muscarinic receptor-stimulated inositol metabolism in cerebellar slices was also age-dependent, being greatest at postnatal day 7 and less pronounced in younger and older animals (Balduini et al. 1991). Following this critical period, alcohol's toxicity to neuronal cells was reduced, implying the acquisition of a protective mechanism.

In conclusion, evidence for alcohol-induced disruption of both intracellular and extracellular molecular cues exists. Although alcohol interferes with all stages of brain development, alcohol at critical times affects more severely the brain regions that are sensitive at the time of exposure.

Alcohol-Induced Neuronal Death

Mechanisms of Neuronal Cell Death

Neuronal cell death appears in two general forms, necrosis or apoptosis, which may be differentiated by their distinct morphological and biochemical changes. Necrotic cell death is marked by a complete loss of ion homeostasis, resulting in cell swelling, degradation of organelle structure, nuclear membrane disruption, and in an eventual rupture of the plasma membrane (Syntichaki & Tavernarakis 2003). Gene transcription, protein synthesis, and respiration halt, while ATP levels rapidly dwindle. With the loss of plasma membrane integrity, neuronal cell contents are discharged into the extracellular compartment, resulting in damage to neighboring cells.

In contrast, apoptotic cell death involves the activation of a series of intracellular signaling pathways for controlled cell demolition. Cells undergoing apoptosis exhibit cell shrinkage and plasma membrane blebbing, while maintaining ion homeostasis and organelle integrity (Yuan & Yankner 2000). Protein synthesis and cell respiration also continue while ATP levels initially remain stable. Unlike in necrosis where considerable proteolytic cleavage by calpains and lysosomal proteases occurs, apoptosis shows greater reliance on caspases which disrupt the cytoskeleton, disable homeostatic and repair processes, and mark the cell for phagocytosis (Bredesen 2000). These processes limit the cellular debris that can damage neighboring cells. Nuclear deoxyribonucleic acid (DNA) fragmentation also occurs (Gavrieli *et al.* 1992). It is important to note that research focusing on either apoptotic or necrotic cell death often uses multiple measures to differentiate these modes of cell death since DNA fragmentation, caspase activation, and phosphatidylserine cell surface expression can also occur in necrotic cell death (Muppidi *et al.* 2004).

Although numerous pathological processes implicate apoptosis in a broad range of neurological conditions including traumatic and ischemic brain injury and many neurodegenerative disorders, precisely orchestrated apoptotic cell death is critical for normal CNS development (de la Rosa & de Pablo 2000). In fact, apoptosis is an evolutionary-conserved and carefully regulated cell suicide mechanism that enables the sculpting of structures in development and the deletion of damaged cells in all metazoans (Muller 2003).

Apoptotic Pathways

Apoptosis may be initiated from extrinsic or intrinsic pathways (Chowdhury *et al.* 2006). Receptor activation at the plasma membrane from specific ligands including cytokines like Fas, interleukin 1- β , or tumor necrosis factor can trigger extrinsic apoptotic pathways that eventually result in caspase activation and cell death. In contrast to these

death receptor-mediated events, the intrinsic pathway involves signals arising from intracellular events that ultimately result in mitochondrial-leakage and the initiation of apoptotic signaling cascades. Caspases that are translated as catalytically inactive zymogens undergo specific proteolytic cleavage for activation (Yakovlev & Faden 2001). In both extrinsic and intrinsic pathways, apoptotic signals are amplified when initiator caspases (-2, -8, -9, and -10) cleave effector caspases (-3, -6, and -7) (Shi 2002).

B-cell lymphoma 2 (BCL-2) oncogene family proteins also play an important role in cell survivability by influencing mitochondrial function (Chao & Korsmeyer 1998). The overall ratio of antiapoptotic to pro-apoptotic BCL-2 family protein levels in a cell determines its susceptibility to apoptotic stimuli (Korsmeyer *et al.* 1993). BCL-2 family proteins include the anti-apoptotic BCL-2 and BCL extra large (BCL-X_L), and the pro-apoptotic BCL-2-associated death promoter (BAD) and BCL-2-associated X protein (BAX) (Chao & Korsmeyer 1998). Artificially altering the expression ratio of these proteins as in overexpressing BCL-2 or in BAX deletion can protect a cell from apoptotic events (Vaux *et al.* 1988, McCurrach *et al.* 1997).

In addition to the transcription levels of BCL-2 family proteins, their posttranslational modification constitutes a significant control step in the regulation of apoptosis. For example, pro-apoptotic BAD, when phosphorylated, is sequestered by cytosolic 14-3-3 proteins (Zha *et al.* 1996). 14-3-3 proteins are regulatory cytosolic proteins that control the subcellular locale and activity of their substrates by complexing with their phosphorylated forms (Fu *et al.* 2000). Dephosphorylation of BAD by calcineurin, a Ca²⁺-dependent phosphatase, causes BAD to disassociate from its regulatory 14-3-3 protein (Wang *et al.* 1999, Springer *et al.* 2000). Consequently, BAD translocates to the outer mitochondrial membrane (Zha *et al.* 1996, Wang *et al.* 1999, Shou *et al.* 2004). In the absence of BAD, BCL-X_L and BCL-2 normally dimerize with BAX (Sedlak *et al.* 1995, Yang *et al.* 1995). Typically, BCL-2 is bound to the outer mitochondrial membrane whereas BCL-X_L may be found in the cytosol or also bound to

mitochondrial membranes (Hsu *et al.* 1997). After BAD's translocation, BAD sequesters BCL-X_L and BCL-2 by forming heterodimers with them, thus freeing pro-apoptotic BAX (Yang *et al.* 1995). Cytosolic BAX then integrates into the outer mitochondrial membrane (Wolter *et al.* 1997), thus initiating cytochrome *c* release from the mitochondria, which in turn leads to caspase activation and apoptosis (Jurgensmeier *et al.* 1998).

How activated BAX increases outer mitochondrial membrane permeability is not clear. BAX, as well as BAD, are bound to cytosolic 14-3-3 proteins. Unlike BAD, BAX mitochondrial translocation may rely on caspase cleavage of 14-3-3 proteins or cytosol acidification, instead of phosphorylation by calcineurin (Nomura *et al.* 2003). Growing evidence suggests that active BAX sequesters and complexes with mitochondrial BCL-2 homologous antagonist/killer (BAK) to form oligomeric pores large enough to transport cytochrome *c* (Korsmeyer *et al.* 2000, Danial & Korsmeyer 2004). Alternately, BAX may directly interact with and trigger opening of the voltage-dependent anion channel (VDAC), also present in the outer mitochondrial membrane (Tsujimoto & Shimizu 2000). The VDAC pore allows nucleotides and small molecules at about 2.6 to 3.0 nm to pass through (Schendel *et al.* 1997). In the absence of BAX, BAK normally complexes with the VDAC (Cheng *et al.* 2003).

The VDAC is a component of the mitochondrial permeability transition pore (MPTP) (Beutner *et al.* 1998), which when open permits molecules up to 1.5 kDa to pass through (Zoratti & Szabo 1995). The MPTP breaches the mitochondrial intermembrane space, thus allowing nonselective traffic to flow between the mitochondrial matrix and the cytosol. In addition to collapsing the mitochondrial voltage potential, the MPTP also admits mitochondrial factors (cytochrome *c*, apoptosis-inducing factor and Smac / Diablo) into the cytosol where they act as caspase cofactors and potentiate apoptosis (Kim *et al.* 2005). Being now permeable to water, mitochondria swell and eventually burst. Although the precise molecular identity and structure of the MPTP is still unclear,

key elements have been identified including the BCL-2 family members as regulatory components and the VDAC (Zorov *et al.* 2009).

Beside regulatory control from the BCL-2 family (Sharpe *et al.* 2004), mitochondrial Ca^{2+} levels also influence the MPTP (Haworth & Hunter 1979, Hunter & Haworth 1979a, Hunter & Haworth 1979b) in that elevated mitochondrial Ca^{2+} levels facilitate MPTP opening (Bernardi & Rasola 2007). Since the mitochondria serve as intracellular Ca^{2+} stores that buffer cellular Ca^{2+} levels, cytosolic Ca^{2+} surges result in mitochondrial Ca^{2+} elevations (Rizzuto & Pozzan 2006, Putney & Thomas 2006). Some groups of mitochondria are strategically situated near inositol 1,4,5 trisphosphate receptors (IP_3Rs), which are located on endoplasmic reticulum (ER) membranes (Otsu *et al.* 1990, Satoh *et al.* 1990). Their presence suggests ER-mitochondrial Ca^{2+} signaling mechanisms.

Indeed, IP_3R activation creates localized elevations in cytosolic Ca^{2+} near mitochondria at concentrations high enough to allow Ca^{2+} entry through the low-affinity mitochondrial Ca^{2+} uniporter (Rizzuto *et al.* 1993, Rizzuto *et al.* 1998). Studies visualizing Ca^{2+} uptake by Ca^{2+} -sensitive compounds targeted to the mitochondria also confirm that Ca^{2+} uptake and mitochondrial loading quickly follows IP_3 -induced Ca^{2+} release from the ER (Hajnoczky *et al.* 2000). Furthermore, IP_3 -linked mitochondrial Ca^{2+} uptake has been observed to drive MPTP opening, as measured by loss of the mitochondria potential and cytosolic cytochrome *c* release (Szalai *et al.* 1999). Illustrating the importance of both IP_3R function and mitochondrial Ca^{2+} uptake in apoptosis, ribonucleic acid interference (RNAi) silencing of $\text{IP}_3\text{R3}$ in chinese hamster ovary cells prevented mitochondria Ca^{2+} uptake and decreases sensitivity to apoptotic stimuli (Mendes *et al.* 2005). Interestingly, translocation of BAD to the mitochondria increased MPTP sensitivity to Ca^{2+} (Narita *et al.* 1998, Pacher & Hajnoczky 2001), which indicates that BCL-2 family apoptotic signals can act synergistically with Ca^{2+} signals originating from the ER to open the MPTP.

Alcohol-Induced Apoptosis in the Fetal Brain

Alcohol exposure in fetal brain during vulnerable periods appears to result in abnormal apoptotic rather than necrotic cell loss. DNA fragmentation (Gavrieli *et al.* 1992) and silver impregnation (DeOlmos & Ingram 1971), hallmarks of apoptosis, increase in various brain regions (Ikonomidou *et al.* 2000) including the cerebellum (Dikranian *et al.* 2005, Light *et al.* 2002) following alcohol exposure in neonatal rats. Elevated caspase 3 activity, another apoptotic measure, increased even after a brief alcohol exposure at 50 mg/dl ethanol for 30 minutes (lower than the federal legal driving limit of 80 mg/dl) throughout the brains of seven day old mice (Young & Olney 2006). This also illustrates the sensitivity of the developing brain to alcohol.

In neuronal cell cultures, alcohol elicits changes strongly suggestive of apoptosis including cell shrinkage, nuclear condensation, DNA fragmentation, increased phosphatidylserine cell surface expression (Annexin-V binding), mitochondrial permeability, and augmented caspase-2, -3, -6, -8, -9 activity (Ramachandran *et al.* 2003, Vaudry *et al.* 2002). One study even assessed both apoptosis and necrosis in alcohol-exposed cerebral explants derived from neonatal rats, finding that ethanol increased apoptosis in a dose-dependent manner without affecting necrosis (Cheema *et al.* 2000). Here, apoptotic cell death was determined by DNA fragmentation and by externalized phosphatidylserine binding; whereas necrosis, by culture media lactate dehydrogenase activity and by externalized phosphatidylserine and propidium iodide binding.

Developmental alcohol exposure has been experimentally observed to alter BCL-2 family regulatory mechanisms to favor apoptosis. In neonatal rat brains, alcohol exposure enhances BAD:BCL-X_L complex formation while diminishing the levels of BAD:14-3-3 complexes, adjustments that favor apoptosis (Han *et al.* 2006, Siler-Marsiglio *et al.* 2006). Artificially altering BCL-2 family expression ratio to inhibit apoptosis, either through BCL-2 overexpression (Heaton *et al.* 1999) or through BAX deficiency (Young *et al.* 2003, Heaton *et al.* 2006), protects the developing cerebellum

from alcohol toxicity. Alcohol administration triggers translocation of BAD (Siler-Marsiglio et al. 2006) and of BAX (Wolter et al. 1997, Adachi *et al.* 2004) to the mitochondria, processes which indicate apoptosis (Siler-Marsiglio et al. 2006). Alcohol encourages BAX binding to VDAC; an interaction which when prevented by the microinjection of anti-VDAC antibodies abolishes alcohol-induced apoptosis (Adachi et al. 2004).

As mentioned before, the VDAC is a component of the MPTP. Other evidence of alcohol's potentiation of MPTP opening has also been experimentally observed. MPTP inhibitor, cyclosporin A, blocks alcohol's induction of MPTP opening, as evidenced by mitochondrial depolarization, mitochondrial swelling, Ca^{2+} and cytochrome *c* release (Pacher & Hajnoczky 2001, Pastorino *et al.* 1999, Higuchi *et al.* 2001, Ramachandran *et al.* 2001, Hajnoczky *et al.* 2005).

It is important to note that BCL-2 family member alterations commonly occur in apoptosis and that the effects described here may not be specific to alcohol-induced neuronal death. Nevertheless, these mechanisms may allow alcohol to sensitize the MPTP to other apoptotic stimuli (Pastorino *et al.* 1999, Ramachandran *et al.* 2001), like Ca^{2+} signals originating from the ER. As mentioned before, BAD mitochondrial localization boosts the Ca^{2+} sensitivity of the MPTP thus increasing the likelihood that Ca^{2+} signals will be apoptotic (Narita et al. 1998, Pacher & Hajnoczky 2001).

Fetal Alcohol Effects on Calcium Signaling

Calcium Homeostasis

In all eukaryotic cells, intracellular calcium levels ($[\text{Ca}^{2+}]_i$) are carefully managed by mechanisms that buffer or sequester Ca^{2+} and prevent spontaneous deviations in its concentration. Since Ca^{2+} plays an important role as a second messenger, maintaining low $[\text{Ca}^{2+}]_i$ is essential to proper cell signaling. Active transport processes of a semi-permeable plasma membrane help sustain a large Ca^{2+} electrochemical gradient, which

are mainly comprised of the plasma membrane Ca^{2+} -ATPase (PMCA) and, in excitable cells (muscle and neuronal), the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX).

The PMCA, which is ubiquitously expressed in all eukaryotic cells (Strehler 1990), removes Ca^{2+} from the cytosol by actively transporting Ca^{2+} against a large transmembrane Ca^{2+} electrochemical gradient. One molecule of ATP is hydrolysed for every calcium ion that is expelled. Although the PMCA shows high affinity for calcium ions ($K_m = 100$ to 200 nM), it extrudes Ca^{2+} at a relatively slow rate (Siegel *et al.* 2006). A feedback mechanism involving Ca^{2+} -bound calmodulin also controls PMCA activity so that high $[\text{Ca}^{2+}]_i$ levels induce greater transport activity (Di Leva *et al.* 2008).

In contrast to the PMCA, the NCX is expressed only in excitable cells that make extensive use of Ca^{2+} signals (Strehler 1990). Rather than ATP hydrolysis, the energy stored in the sodium (Na^+) electrochemical gradient drives the removal of Ca^{2+} . One Ca^{2+} ion is expelled in exchange for the import of three Na^+ ions. Unlike the PMCA, the NCX displays low affinity for Ca^{2+} , but instead transports Ca^{2+} ions at a high rate (five thousand Ca^{2+} ions per second) (Carafoli *et al.* 2001). The net effect of these plasma membrane mechanisms is to create an environment of low cytosolic Ca^{2+} (50 to 200 nM) relative to the extracellular space (1 mM) (Raza *et al.* 2007, Putney 1997).

Organelles such as the endoplasmic reticulum (ER), the mitochondria, and the nucleus also withdraw Ca^{2+} from the cytosol and serve as Ca^{2+} intracellular stores. The ER uses a Ca^{2+} -ATPase similar to the PMCA to sequester Ca^{2+} from the cytosol, the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) (Strehler & Zacharias 2001) against a high Ca^{2+} gradient. Unlike PMCA regulation, Ca^{2+} -calmodulin binding plays no role in controlling SERCA pump activity (Di Leva *et al.* 2008).

Uptake of cytosolic Ca^{2+} into the lumen allows the ER to act as an intracellular Ca^{2+} storage site. Low-affinity Ca^{2+} -binding proteins within the ER lumen, like calsequestrin in muscle and chaperones like calreticulin, further enhance the Ca^{2+} holding capacity (Pozzan *et al.* 1994). The ER's Ca^{2+} pool serves multiple ends. Normal

chaperone function requires high Ca^{2+} levels and is thereby crucial for proper protein folding (Michalak *et al.* 2002). The rapid uptake of Ca^{2+} by SERCA pumps increases cytosolic Ca^{2+} buffering capacity and efficiency. Lastly, the ER is a source for intracellular Ca^{2+} in many signaling pathways.

After the ER, the mitochondria are the next most significant site of intracellular Ca^{2+} storage. Although it has been known since the 1950's that mitochondria accumulate Ca^{2+} (Carafoli 2003), the molecular basis of mitochondrial Ca^{2+} accumulation is still controversial. During ATP synthesis, normally respiring mitochondria pump protons out of the mitochondrial matrix into the intermembrane space, thus generating a strong proton gradient across the inner membrane (Mitchell 1961). This results in a steep mitochondrial membrane potential which also provides energy for Ca^{2+} uptake via the mitochondrial calcium uniporter (MCU) located on the inner mitochondrial membrane (Miller 1998). Although resting $[\text{Ca}^{2+}]_i$ levels are below the MCU affinity values for Ca^{2+} , some groups of mitochondria are strategically located near where Ca^{2+} ions are released from the ER (Rizzuto *et al.* 1998), so that local Ca^{2+} concentrations exceeds MCU affinity values and therefore are above the threshold for uptake (Rizzuto *et al.* 1992). The close proximity of mitochondria near the ER enables the propagation of ER Ca^{2+} signals into the mitochondria.

To reach the MCU on the inner mitochondrial membrane, cytosolic Ca^{2+} must transverse the outer mitochondrial membrane through the VDAC (Mannella 1982). The VDAC exists in either an open-state with high conductance and weak anion selectivity or a closed-state with low conductance and weak anion selectivity. The mitochondrial membrane potential determines VDAC gating so that higher and lower membrane potentials facilitate respectively the open- and the closed-state (Rostovtseva *et al.* 2005). Although Ca^{2+} does not affect VDAC gating, the "closed-state" augments Ca^{2+} uptake (Tan & Colombini 2007).

Mitochondrial traffic of Ca^{2+} is not unidirectional. Mitochondrial Ca^{2+} efflux normally occurs via the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (mNCX) (Palty *et al.* 2010) and the mitochondrial $\text{H}^+/\text{Ca}^{2+}$ exchanger (mHCX) located on the inner mitochondrial membrane. For both exchangers, the Ca^{2+} gradient across the inner mitochondrial membrane provides energy for counter-transport although evidence exists for supplemental sources (Pfeiffer *et al.* 2001). The mNCX is electrogenic, importing 3 Na^+ ions for every Ca^{2+} ion exported (Baysal *et al.* 1994), whereas the mHCX is electroneutral, importing 2 H^+ for each Ca^{2+} ion (Gunter *et al.* 2000) and predominates in non-excitabile cells. Rapid mitochondrial Ca^{2+} influx can saturate these efflux pathways (Bernardi 1999), elevating mitochondrial Ca^{2+} levels. This can subsequently stimulate the opening of the mitochondrial permeability transition pore (MPTP) (Massari & Azzone 1972). MPTP opening disrupts mitochondrial voltage potentials and permeabilizes mitochondrial membranes to molecules up to 1500 Da (Zoratti & Szabo 1995), thus allowing mitochondrial contents to spill out.

The signaling mechanisms that utilize Ca^{2+} are many and can mobilize Ca^{2+} from either extracellular or intracellular sources. One ubiquitous signaling mechanism involves ligand activation of G_q -linked receptors to stimulate phospholipase C (PLC) to release Ca^{2+} from intracellular stores (Cockcroft & Gomperts 1985). PLC promotes phosphoinositide turnover, thus elevating cytosolic inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) levels (Berridge & Irvine 1989). DAG activates protein kinase C (PKC), whereas IP_3 is responsible for release of Ca^{2+} from intracellular stores. IP_3 induces Ca^{2+} release from intracellular stores (Furuichi & Mikoshiba 1995) by activating the inositol 1,4,5 trisphosphate receptor (IP_3R) localized on ER membranes (Vermassen *et al.* 2004). Interestingly, cytosolic Ca^{2+} levels also control IP_3R activity in that low Ca^{2+} levels sensitize the receptor to IP_3 while higher levels are inhibitory (Hajnoczky & Thomas 1997).

IP₃Rs typify one major efflux mechanism from the ER and are comprised of four subunits, each with six transmembrane segments (Yoshida & Imai 1997). Although there are reports of heterotetramers forming (Joseph *et al.* 2000), IP₃Rs mainly exist as homotetramers composed of any of three distinct isoforms that undergo alternative splicing (Patel *et al.* 1999). The receptor complex has four IP₃ binding sites (one per subunit), of which three must be occupied for the channel to remain in a stable open configuration (Marchant & Taylor 1997).

Like IP₃Rs, ryanodine receptors (RyRs) also located on the ER membrane represent another method for ER Ca²⁺ efflux. RyRs share the homotetrameric configuration and considerable structural homology to IP₃Rs (Iino 1999). Unlike IP₃Rs, the physiological ligand for RyRs are Ca²⁺ ions, which stimulate Ca²⁺ release from the ER by binding on its cytosolic side (Zucchi & Ronca-Testoni 1997). Therefore a small amount of cytosolic Ca²⁺ near the receptor can induce the release of even more Ca²⁺, establishing a positive feedback loop. As such, RyRs are considered a calcium-induced calcium release (CICR) Ca²⁺ channel (Fabiato 1983).

Other signaling mechanisms utilizing Ca²⁺ entry are ligand-gated ion channels like NMDA receptor and voltage dependent Ca²⁺ channels.

Elevation of Intracellular Calcium Levels

Alcohol's effects on Ca²⁺ signaling have long been known. Electrophysiological studies in the 1980s performed in mollusks demonstrated that alcohol reduced voltage-sensitive Ca²⁺ currents at physiological doses by enhancing the inactivation rate of the Ca²⁺ current (Camacho-Nasi & Treistman 1986, Oyama *et al.* 1986). The authors of these studies postulated that elevated intracellular Ca²⁺ levels may be responsible as alcohol failed to affect barium ion currents carried by the same channels. This effect on Ca²⁺ uptake was also replicated in depolarized rat and mouse brain-derived synaptosomes (Harris & Hood 1980) and cultured PC12 cells (Messing *et al.* 1986). In *Xenopus*

oocytes, acute alcohol exposure initiated a Ca^{2+} -dependent chloride conductance, an effect blocked by the intracellular administration of the Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA) (Wafford *et al.* 1989). Adding EGTA to the media or eliminating the extracellular Ca^{2+} failed to block the alcohol-initiated chloride conductance, suggesting that alcohol stimulated an intracellular source of Ca^{2+} .

More direct evidence of alcohol's effects on intracellular Ca^{2+} may be found with Ca^{2+} -sensitive fluorescent dyes. Studies using Fura-2 in mouse (Daniell *et al.* 1987) and rat (Rezazadeh *et al.* 1989) brain synaptosomes found that alcohol raised intracellular calcium ($[\text{Ca}^{2+}]_i$) levels. Although the basal $[\text{Ca}^{2+}]_i$ depended on the extracellular Ca^{2+} concentration of the media, alcohol's elevation of $[\text{Ca}^{2+}]_i$ was independent of the extracellular Ca^{2+} concentration, indicating that the Ca^{2+} elevations originated from an intracellular source. One study in rat brain synaptosomes simultaneously measured $[\text{Ca}^{2+}]_i$ by Fura-2 fluorescence and Ca^{2+} intake by the uptake of extracellular $^{45}\text{Ca}^{2+}$, and found that alcohol increased $[\text{Ca}^{2+}]_i$ while decreasing Ca^{2+} uptake (Davidson *et al.* 1988). This also discounts the possibility that the elevations arise from an extracellular source.

Alcohol's induction of intracellular Ca^{2+} has also been observed in living systems. Alcohol elevated Ca^{2+} levels in neonatal rat astrocyte cultures (Holownia *et al.* 1997, Hirata *et al.* 2006), in mouse preimplantation embryos (Stachecki & Armant 1996) and in chick neural crest (Debelak-Kragtorp *et al.* 2003, Garic-Stankovic *et al.* 2005).

Enhanced Phospholipase C Activity

Considerable evidence substantiates that acute alcohol exposure raises cytosolic Ca^{2+} levels by stimulating PLC to release Ca^{2+} from intracellular stores. PLC promotes phosphoinositide turnover, thus elevating cytosolic IP_3 and DAG levels (Berridge & Irvine 1989). IP_3 in turn activates IP_3Rs localized on ER membranes to release Ca^{2+} from intracellular stores (Furuichi & Mikoshiba 1995).

The evidence for alcohol's role in PLC signaling originates from alcohol's effects on PLC products (DAG, IP₃, and Ca²⁺). A study using the fluorescent dye, Indo-1, to monitor Ca²⁺ release from mouse brain microsomes, found that the alcohol-induced Ca²⁺ surges resembled those triggered by IP₃ (Daniell & Harris 1989). In addition to stimulating Ca²⁺ release in rat brain microsomes (Machu *et al.* 1989), alcohol elicited the release of PLC-specific products from a synthetic PLC substrate (Natsuki & Yamaguchi 1996). In rat hepatocytes, alcohol raised cytosolic Ca²⁺ and IP₃ levels while lowering phosphatidylinositol 4,5-bisphosphate levels (Hoek *et al.* 1987, Reinlib *et al.* 1990). Acute alcohol exposure also increased DAG production both in mouse brain tissue and neuroblastoma cell cultures (Katsura *et al.* 1994). In all these studies, acute alcohol exposure elevated the production of the PLC products: DAG, IP₃, and Ca²⁺ *in vitro*. *In vivo*, the pharmacological inhibition of PLC also blocked alcohol-dependent Ca²⁺ release from intracellular ER stores in mouse (Stachecki & Armant 1996) and chick embryos (Debelak-Kragtorp *et al.* 2003).

How alcohol stimulates PLC activity is unclear. One possibility is that alcohol alters G-protein dynamics upstream of PLC to promote greater GTP / lower GDP affinity of the α subunit, encouraging its dissociation from the $\beta\gamma$ subunit. Indeed, alcohol's augmentation of $\beta\gamma$ activity has been demonstrated by enhancement of PLC-mediated phosphoinositid breakdown (Rooney *et al.* 1989), by increased activity of $\beta\gamma$ -linked protein kinase A (Lewohl *et al.* 1999), and by stimulation of G protein-coupled inwardly rectifying potassium channels (Yao *et al.* 2003, Yao *et al.* 2002). Inhibition of G_{i/o}-protein dissociation (to block G $\beta\gamma$ subunit activity), either by pertussis-induced ADP ribosylation of G $\alpha_{i/o}$ or by obstruction of GDP-GTP exchange with suramin analog, NF023, prevents the intracellular Ca²⁺ elevations associated with alcohol stimulation of G $\beta\gamma$ -mediated PLC signaling (Garic-Stankovic *et al.* 2005).

Stimulation of Inositol 1,4,5 Trisphosphate Receptors

Alcohol-induced elevations in cytosolic Ca^{2+} require downstream activation of the IP_3 receptor (IP_3R) via PLC signaling. IP_3 generated from elevated PLC activity binds and activates the IP_3R , initiating Ca^{2+} release from ER stores (Ma *et al.* 2000, Furuichi & Mikoshiba 1995). The IP_3R 's role in alcohol toxicity is substantiated by the following evidence: 1) Pharmacological inhibition of PLC (Stachecki & Armant 1996, Debelak-Kragtorp *et al.* 2003, Garic-Stankovic *et al.* 2005), but not PKC (Stachecki & Armant 1996), prevents alcohol-dependent Ca^{2+} release from intracellular ER stores. Since PKC is the downstream target for DAG, this implies that alcohol elevates IP_3 levels to increase cytosolic Ca^{2+} . 2) In gastric mucosal cell lines (Miller *et al.* 2001, Kokoska *et al.* 1999) and rat hepatocytes (Hoek *et al.* 1987, Reinlib *et al.* 1990), alcohol induces concomitant increases for both cytosolic IP_3 and Ca^{2+} , which is consistent with IP_3 activation of the IP_3R and subsequent Ca^{2+} release from ER stores. 3) Lastly, xestospongin C, an allosteric membrane-permeable IP_3R inhibitor (Oka *et al.* 2002, Ozaki *et al.* 2002), blocks alcohol-induced elevations of cytosolic Ca^{2+} but not IP_3 levels (Garic-Stankovic *et al.* 2005), signifying that IP_3R activation is necessary for ethanol-induced Ca^{2+} spikes.

IP_3R -mediated elevations in intracellular Ca^{2+} represent a key apoptotic mechanism in alcohol toxicity. Disruption of PLC signal transduction, either by pharmacological interference with G-Proteins or PLC, obstructs both ethanol-related Ca^{2+} releases from ER stores and hinders apoptotic cell death (Garic-Stankovic *et al.* 2005). This indicates that both alcohol-induced cytosolic Ca^{2+} elevations and toxic effects are a consequence of PLC signal transduction. In another study that mobilized Ca^{2+} to induce apoptosis in cultured rat astrocytes, larger increases in cytosolic Ca^{2+} correlated with higher percentages of cell death (Hirata *et al.* 2006). Alcohol-induced cellular damage in gastric cell lines, as measured by ethidium homodimer and trypan blue dye exclusion, correlates to greater intracellular Ca^{2+} accumulation (Miller *et al.* 2001, Kokoska *et al.* 1999). Depletion of intracellular Ca^{2+} stores, either by chelation with

ethyleneglycotetraacetic acid (EGTA) (Debelak-Kragtorp et al. 2003) or by thapsigargin pretreatment (Kokoska et al. 1999), also prevents alcohol-induced intracellular Ca^{2+} currents and cell death. Collectively, these studies link alcohol's toxic effects to cytosolic Ca^{2+} elevations from ER stores.

These findings also suggest that IP_3R antagonism should protect against alcohol toxicity. RNAi silencing of IP_3 receptor, type III, ($\text{IP}_3\text{R3}$) indeed reduces the sensitivity of chinese hamster ovary cells to numerous apoptotic stimuli (Mendes et al. 2005). IP_3R antagonists, 2-APB (van Rossum *et al.* 2000) and enoxaparin (Jonas *et al.* 1997), prevent glutamate-induced Ca^{2+} releases and neurotoxicity in rat cerebellar slices (Tang *et al.* 2005). Addition of the IP_3R antagonist, xestospongine C (Gafni *et al.* 1997), to primary neuronal cultures hinders apoptotic cell death triggered by glycolysis inhibition (Hernandez-Fonseca & Massieu 2005) and β -amyloid (Ferreiro *et al.* 2004). Xestospongine C also obstructs cell death in apoptosis-susceptible mouse embryonic fibroblast cultures lacking nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$) (Camandola *et al.* 2005). These studies collectively demonstrate that IP_3R antagonism can provide a protective effect from apoptotic stimuli.

However, in the context of alcohol-induced neuronal death, the effects of IP_3R antagonism have not been consistent. In the developing neural crest of chicken embryos, although xestospongine C blocks alcohol-induced cytosolic Ca^{2+} increases, it also promotes cell death (Garic-Stankovic et al. 2005). *In vivo*, it has been suggested that xestospongine C may hinder essential PLC-mediated trophic support, thereby promoting apoptosis within the neural crest (Garic-Stankovic et al. 2005). Indeed, PLC-mediated IP_3 release is necessary for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) induction of neuronal differentiation in rat embryonic brain cells (Widmer *et al.* 1993, Widmer *et al.* 1992). Ethanol, via PLC-dependent mechanisms, elevates cytosolic Ca^{2+} well above endogenous levels in the developing neural crest to trigger apoptotic cell

death (Debelak-Kragtorp et al., 2003). These IP₃R-mediated Ca²⁺ elevations are likely greater than those induced through neurotrophic support.

Fetal Alcohol Effects on Nitric Oxide Signaling

The Nitric Oxide Signaling Pathway

Nitric oxide (NO) is a small diffuse gas that functions as an important signaling molecule and has been implicated in numerous physiological and pathological processes. NO's discovery as a signaling molecule stems from its role in arterial vasodilation and thus was previously known as endothelium-derived relaxing factor (EDRF) before the recognition of its molecular identity (Palmer *et al.* 1987, Ignarro *et al.* 1987, Furchgott 1987). Within the nervous system, NO serves both as a neurotransmitter and as a modulator of ligand-gated receptors. Due to its physical properties, NO diffuses readily and widely and even into cells not directly connected by a synapse (Boehning & Snyder 2003). Even so, its short half-life limits its effective range (Ignarro 1990). Cells can neither store nor sequester NO to control the quantity released by the cell and instead must rely on regulating its production.

NO synthesis depends on controlling the expression, activation, and cellular and sub-cellular locale of nitric oxide synthase (NOS) (Abbott & Nahm 2004). In the presence of nicotinamide adenine dinucleotide phosphate (NADPH), and cofactors flavin adenine dinucleotide and tetrahydrobiopterin, NOS oxidizes the amino acid, L-arginine to generate L-citrulline and NO (Moncada 1993). At least three major NOS isoforms exist arising from the transcription of three different gene products, which differ in their tissue distribution and activation properties. These are type 1, neuronal NOS (nNOS); types 2, inducible NOS (iNOS); and type 3, endothelial NOS (eNOS). Both neuronal and endothelial NOS are constitutively expressed in their eponymous tissues, requiring Ca²⁺ calmodulin for activation (Schmidt *et al.* 1992). Instead of Ca²⁺ calmodulin regulation,

iNOS activity is controlled by transcription, and is localized to macrophages and smooth muscle (Berdeaux 1993).

Neuronal NOS is strongly expressed in the cerebellum with high levels found in cerebellar granule cells, basket and stellate cells (Schilling *et al.* 1994) and normally no expression in Purkinje cells (Crepel *et al.* 1994, Gotti *et al.* 2005, Bredt *et al.* 1990). NOS expression begins on postnatal day 7 in the mouse cerebellum (Giuli *et al.* 1994). However, tissue injury may induce nNOS expression in Purkinje cells (Saxon & Beitz 1994). There is also evidence linking to long term depression (LTD) to nNOS expression in the cerebellum in that Purkinje cells from transgenic mice lacking a functional nNOS gene (nNOS^{-/-}) do not experience LTD (Lev-Ram *et al.* 1997, Linden *et al.* 1995).

The NO generated by NOS, stimulates soluble guanylyl cyclase (sGC) to convert guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) (Moncada & Higgs 1995). Membrane-bound guanylyl cyclase also generates cGMP but does not respond to NO (Pyriochou & Papapetropoulos 2005). In turn, cGMP, by binding to the regulatory subunits of protein kinase G (PKG), activates PKG, thus allowing it to phosphorylate multiple substrates at serine/threonine sites (Francis & Corbin 1999). In mammals, two genes code for PKG, type 1 (cGK1) and type 2 (cGKII), for which two type 1 isoforms exist as the result of alternative splicing: cGKI α and cGKI β (Hofmann *et al.* 2009).

It is important to note the NO signaling pathway (NOS \rightarrow NO \rightarrow sGC \rightarrow cGMP \rightarrow PKG) can only function if all components are expressed and active within the same locale. For example, despite the lack of nNOS in adult Purkinje cells, they express high levels of sGC (Ariano *et al.* 1982) which can be stimulated by the NO produced in neighboring cells. Within the developing mouse cerebellum, sGC expression begins in the external germinal cell layer on postnatal day 3, and in the molecular, Purkinje, and granule cell layers from postnatal day 7 onwards with the heaviest sGC expression occurring in the Purkinje cell layer for the adult mouse (Giuli *et al.* 1994). In the adult

mouse, cGKI α and cGKI β also show strong and weak cerebellar expression, respectively (Geiselhoringer *et al.* 2004a) with cGKI α strongly expressed in Purkinje cells and the deep cerebellar nuclei (Feil *et al.* 2005). Low levels of cerebellar cGKII can also be found in the adult rat cerebellum (el-Husseini *et al.* 1995).

Nitric Oxide Signaling in Fetal Alcohol Spectrum

Disorders

Within the developing cerebellum, activation of glutamate NMDA receptors provide an important neurotrophic stimulus (Balazs *et al.* 1988, Balazs *et al.* 1989, Burgoyne *et al.* 1993) that relies on NO signaling (Pantazis *et al.* 1998). Glutamate NMDA receptors bring Ca²⁺ into the cell which then activates Ca²⁺-dependent nNOS (Bredt & Snyder 1989, Southam *et al.* 1991). By this same mechanism (through NO signaling), NMDA activation also diminishes alcohol neurotoxicity (Pantazis *et al.* 1998).

Evidence for this pathway stems from *in vitro* cell culture studies and *in vivo* studies using transgenic mice. *In vitro*, NO mimics NMDA in reducing alcohol-induced cell loss in cerebellar granule neuron (CGN) cell cultures. Blocking nNOS or sGC or PKG downstream abolishes this neuroprotective effect, indicating that NMDA-mediated neuroprotection to alcohol-induced cell loss depends on NO signaling (Pantazis *et al.* 1998, Bonthius *et al.* 2004). In CGN cultures derived from mice with a null mutation for nNOS (nNOS^{-/-}), alcohol induced significantly greater cell loss than in cultures derived from wild-type mice (Bonthius *et al.* 2002). *In vivo*, nNOS^{-/-} mice also demonstrate more vulnerability to alcohol toxicity than wild-type mice (Bonthius *et al.* 2002), in that nNOS^{-/-} mice exhibit greater alcohol-induced cerebellar and hippocampal damage than wild-type mice. These mice also fail to develop any alcohol resistance, suggesting that NO signaling is vital in countering alcohol toxicity.

In wild-type mice (Giuli *et al.* 1994) and rats (Wang *et al.* 1998), cerebellar NOS expression begins on postnatal day 7, marking the end of a period of great alcohol

susceptibility. It follows that alcohol susceptibility during early postnatal life may be linked to a relative deficiency of nNOS. CGN cell culture studies reinforce this assertion in that: 1) pharmacological blockade within the NO signaling pathway prevents alcohol resistance, and 2) additional downstream stimulation restores resistance (Bonthius et al. 2004). That NOS inhibition in adult rats also exacerbates alcohol-induced neurodegeneration (Zou *et al.* 1996), further supports a protective role for NO against alcohol toxicity.

Interestingly, alcohol suppresses NO signaling by reducing Ca^{2+} -dependent NOS activity (Hoffman et al. 1989b, Hoffman *et al.* 1989a). Alcohol reduces tyrosine phosphorylation of NMDA receptor $\text{NR}_{2A/B}$ subunits, thus attenuating NMDA-mediated uptake of extracellular Ca^{2+} in CGN cultures (Ferrani-Kile *et al.* 2003). Conversely, NMDA receptor activation confers alcohol resistance by stimulating Ca^{2+} -dependent NOS activity in postnatal CGN cultures (Pantazis et al. 1998, Pantazis *et al.* 1995). These two studies, when considered in tandem, indicate that increased NOS activity provides neuroprotection. However, since chronic alcohol ingestion damages brain regions regardless of NOS's presence, it is unlikely that NOS inhibition represents a primary mechanism for ethanol neurotoxicity (Zou et al. 1996).

The NO signaling pathway is neuroprotective in other contexts (Fiscus 2002). The addition of NO donors prolongs the survival of cultured sympathetic neurons deprived of nerve-growth factor (Farinelli *et al.* 1996) and inhibits apoptosis in serum-deprived PC12 (rat adrenal tumor) cultures (Kim *et al.* 1999b). Additionally, soluble amyloid precursor protein improves neuronal survival rates and lowers cytosolic Ca^{2+} concentrations by raising cyclic guanosine monophosphate (cGMP) levels in rat hippocampal neurons experiencing glutamate excitotoxicity or glucose deprivation (Barger *et al.* 1995). Blocking PKG downstream also eliminates soluble amyloid precursor protein protective effects (Barger et al. 1995). Overall, these data suggest that

NO signal transduction represents a convergence point for multiple neuroprotective pathways.

How NO signaling counters apoptotic mechanisms remains poorly understood. Since dysregulation of Ca^{2+} signaling commonly triggers apoptosis, NO may aid in regulating intracellular Ca^{2+} levels.

Neuroprotection through Nitric Oxide Signaling

NO-mediated ethanol resistance likely involves inhibiting MPTP opening despite the presence of apoptotic stimuli. NO's relationship to cell death is complex, imparting both pro- and anti-apoptotic effects. Whether NO promotes cell survival or death depends on multiple factors including the concentration and source of NO (Kim *et al.* 1999a). For neuronal cells, the primary endogenous source of NO is nNOS, shown as neuroprotective under multiple contexts (Bonthius *et al.* 2002, Keilhoff *et al.* 2004).

NO's obstruction of MPTP opening has been previously demonstrated (Brookes *et al.* 2000, Dedkova & Blatter 2005). In these studies, NO donors reduced mitochondrial swelling, as well as prevented the release of cytochrome c and fluorescent probes targeted to the mitochondria, consistent with MPTP gating inhibition (Brookes *et al.* 2000, Dedkova & Blatter 2005). NO signaling may possibly halt apoptosis by hindering the passage of Ca^{2+} from the ER to the mitochondria.

IP_3R activation triggers both Ca^{2+} release from ER stores (Furuichi & Mikoshiba 1995) and mitochondrial Ca^{2+} uptake (Hajnoczky *et al.* 2000). Since IP_3Rs contain PKG phosphorylation sites (Komalavilas & Lincoln 1994, Haug *et al.* 1999) and comprise a major conduit for Ca^{2+} release from intracellular stores (Furuichi & Mikoshiba 1995), it is likely that IP_3R phosphorylation by PKG constitutes an additional regulatory mechanism in Ca^{2+} release from the ER. IP_3Rs may also represent a target for NO-induced ethanol resistance, being both strategically located and functionally downstream of PKG. A physiological role for NO-mediated control of IP_3Rs in mediating ethanol

resistance is also supported in that IP₃Rs are heavily expressed in ethanol-susceptible Purkinje neurons (Ross *et al.* 1992).

IP₃R phosphorylation by PKG was first described using tissues derived from aortic smooth muscle (Komalavilas & Lincoln 1994, Koga *et al.* 1994, Komalavilas & Lincoln 1996), platelets (el-Daher *et al.* 1996), and cerebellum (Koga *et al.* 1994, Haug *et al.* 1999). PKG phosphorylates two serines within IP₃R (Haug *et al.* 1999). Inhibition of agonist-induced intracellular Ca²⁺ currents by PKG has been described in a variety of cell systems including smooth muscle (Murthy & Makhlof 1995, Murthy *et al.* 1993, Murthy & Zhou 2003), megakaryocytes (Tertyshnikova *et al.* 1998), and more recently in neural tissue (Fernandez *et al.* 2005, Morales *et al.* 2005). These findings suggest that PKG activity attenuates IP₃R Ca²⁺ conductance by phosphorylation.

IP₃Rs in Nitric Oxide-Mediated Neuroprotection

Interestingly, PKG potentiation of IP₃R-mediated intracellular Ca²⁺ currents has also been observed in hepatocytes (Guihard *et al.* 1996, Rooney *et al.* 1996). One study (Wagner *et al.* 2003), in order to investigate the effects of IP₃R phosphorylation in a null background, transfected cell lines devoid of endogenous IP₃R with IP₃ receptor, type I (IP₃R1). Selective mutation of these phosphorylation sites in IP₃R1 abolished PKG-induced Ca²⁺ currents (Wagner *et al.* 2003, Wagner *et al.* 2004). These findings appear to conflict with the notion that PKG phosphorylation universally inhibits IP₃R function.

A rationale for this discrepancy may involve the IP₃R isoforms under study. Presently, three isoforms of IP₃Rs have been cloned, denoted as types I, II, and III (Taylor *et al.* 1999). The IP₃R1 isoform undergoes alternative splicing, allowing for long and short forms (Danoff *et al.* 1991). The long form is the predominant splice variant found in adult neuronal tissues, whereas the short form is mainly expressed in fetal brain and peripheral tissues (Danoff *et al.* 1991). Unlike the long form of the IP₃R1 (Nakade *et al.* 1994), phosphorylation of the short form reduces IP₃-induced Ca²⁺ release

(Tertyshnikova & Fein 1998, Tertyshnikova et al. 1998). Therefore, functional different tissue specific splice variants may explain the observed inconsistencies in the effects of IP₃R1 phosphorylation by PKG. Studies that found phosphorylation-induced inhibition of IP₃R activity likely dealt with the short form of the IP₃R1.

Another plausible explanation for this discrepancy links NO modulation of IP₃R function to expression of IP₃ receptor-associated cGMP kinase substrate (IRAG), also found on ER membranes (Schlossmann *et al.* 2000). IRAG forms functional complexes with PKG-I β and IP₃R1 to decrease Ca²⁺ currents (Schlossmann et al. 2000), but not with PKG-I α or PKG-II (Ammendola *et al.* 2001). Since IRAG mRNA antisense oligonucleotides block NO inhibition of IP₃R1-mediated Ca²⁺ release, this suggests that IRAG expression is a precondition at least for PKG-I β and IP₃R1 interactions (Fritsch *et al.* 2004, Geiselhoringer *et al.* 2004b).

For IRAG to confer ethanol-resistance, IRAG must inhibit ER-mitochondrial Ca²⁺ signaling by complexing with the PKG and IP₃R isoforms available in vulnerable brain regions. IP₃R1, IP₃R3, but not IP₃ receptor, type II, (IP₃R2) cerebral distribution (Sharp *et al.* 1999, Furuichi *et al.* 1990, Newton *et al.* 1994) mirror the regions of ethanol-susceptibility, showing high cerebellar and hippocampal expression. Since central nervous system IP₃R2 expression is limited to spinal cord glia (Newton et al. 1994, Sharp et al. 1999), it cannot be responsible for ethanol-induced apoptosis. In adult mice, PKG-I β expression is high in the hippocampus and olfactory bulb, but low in the cerebellum (Geiselhoringer et al. 2004a). Since IRAG's cerebral distribution in adult mice is limited to the thalamus (Geiselhoringer et al. 2004a) and does not co-localize with PKG-I β in the same brain regions, these data suggest that NO-mediated ethanol resistance may not involve IRAG.

An *In vitro* Model of Fetal Alcohol Syndrome Disorders

The Need for In Vitro Models

Although *in vivo* animal models of FASD lend themselves well to characterizing neurostructural effects in brain regions and the conditions which modulate their severity, studying intracellular mechanisms associated with alcohol-induced cell death requires a higher level of experimental control than that afforded by *in vivo* models. Cell loss in specific neuronal populations depends on the dose and the pattern of alcohol exposure in addition to the innate vulnerability of the cell population (West 1987, West et al. 1990). Additional factors can also influence the variable being measured, including the local environment, maturity, synaptic connectivity, and glial support. Using an *in vivo* model to parse out each of these components and weigh their respective contribution is impractical, especially when intracellular signaling is being investigated.

A simpler biological system can be achieved with a cell culture model which allows precisely controlled experimental conditions and limits potential interacting variables. However, one serious area of concern when using *in vitro* cell culture models is one of relevance in that the effects being observed may potentially have no physiological bearing *in vivo*. This is why the rationale and design of *in vitro* studies are usually driven by observations made in *in vivo* studies.

Cerebellar Granule Cell Cultures

Primary cultures of murine CGN are a particularly advantageous cell culture model for studying mechanisms of alcohol toxicity in FASD, being a well-recognized and characterized model system for molecular and cell biological studies of neuronal development and intracellular signaling pathways. CGN preparations readily yield millions of granule neurons in relatively homogenous cell cultures (about 95% pure) (Dutton 1990). Limiting glia and other non-neuronal cell culture contamination through this preparation prevents the complications associated from response inconsistencies

between different cell types. Since CGN cultures consist of primary cells, studies using CGN cultures have greater physiological relevance to *in vivo* neurons and conditions than those conducted in neoplastic cell lines.

As mentioned, neonatal alcohol exposure in mice severely depletes cerebellar neurons, thus indicating the extreme susceptibility of the developing cerebellum to alcohol (Goodlett et al. 1990). In particular, neonatal alcohol exposure severely diminishes CGN cell numbers, hence demonstrating their vulnerability as a cell population (Pierce *et al.* 1989). Extracting CGNs when the cerebellum is vulnerable to alcohol exposure enhances the relevance to fetal alcohol physiology in this cell culture model system. The alcohol vulnerability has been further demonstrated *in vivo*, since alcohol reduces surviving CGN numbers in dose-dependent manner in culture (Pantazis et al. 1993). This has been attributed to cell death since CGN in these cell preparations do not proliferate (Pantazis et al. 1993).

Rationale and Dissertation Overview

Prior studies have demonstrated that the NO signaling pathway (NOS → NO → sGC → cGMP → PKG) diminishes alcohol's toxic effects, consequently reducing neuronal loss both *in vivo* and *in vitro* (Bonthius et al. 2004, Bonthius *et al.* 2003, Bonthius et al. 2002, Pantazis et al. 1998). How PKG mitigates neuronal cell death resulting from neonatal alcohol exposure is unknown. As mentioned earlier, alcohol exposure during neurogenesis induces abnormally high apoptotic neuronal death across multiple brain regions, including in the cerebellum. ER release of Ca²⁺, a key apoptotic mechanism (Pinton *et al.* 2008), requires the IP₃R, a known PKG substrate (Komalavilas & Lincoln 1994, Haug et al. 1999). In this dissertation, the central hypothesis has been that as neonatal alcohol exposure disturbs Ca²⁺ signaling to excessively trigger apoptosis, PKG stimulation counters it, likely by reducing ER release of Ca²⁺. To assess this hypothesis, these studies focused on a crucial intersection point, the IP₃R, where NO

signaling pathways can influence Ca^{2+} -mediated apoptotic mechanisms using a CGN cell culture model.

This dissertation is divided into two main parts. To comprehend how PKG ameliorates alcohol toxicity, better understanding of how alcohol achieves cell death was necessary. Part one (chapter II) is geared toward identifying the mechanisms responsible for alcohol's effects upon intracellular Ca^{2+} signaling. These experiments tested the hypothesis that the alcohol initiates neuronal cell death by enhancing intracellular Ca^{2+} signaling through ER-dependent pathways. Although previously mentioned studies demonstrated the involvement of the $\text{PLC} \rightarrow \text{IP}_3 \rightarrow \text{IP}_3\text{R} \rightarrow \text{Ca}^{2+}$ pathway in neurodevelopmental alcohol toxicity (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2010, Garic-Stankovic et al. 2006, Garic-Stankovic et al. 2005), these studies were performed *in vivo* with chick embryos under conditions less amendable to experimental manipulation. In contrast, our experiments were performed *in vitro* with CGN cell cultures derived from alcohol-vulnerable 5 to 7 day-old mice, a model system that allows for greater control of experimental conditions pertaining to the examination of intracellular signaling pathways.

Using CGN cultures, our studies examined alcohol-induced effects on intracellular Ca^{2+} levels and on neuronal cell death following pharmacological manipulations upstream and downstream from the IP_3R , and identify the source of the alcohol-induced intracellular Ca^{2+} elevation. Temporal manipulation of protective agents in relation to alcohol exposure also demonstrated that alcohol's lethality is confined to the moments of initial exposure. Lastly, the reliance of alcohol-induced neuronal death on a small selection of downstream apoptotic interactants was examined by inhibiting calcineurin and CaMKii in the cytosol and the MCU and MPTP in the mitochondria.

Part two (chapter III) examines the relationship between neuroprotective NO signaling and the disruption of intracellular Ca^{2+} that accompanies alcohol exposure. Since intracellular Ca^{2+} disruptions commonly trigger cell death, and PKG downstream

can regulate $[Ca^{2+}]_i$, this study examined the hypothesis that PKG activation ameliorates alcohol toxicity by preventing alcohol-induced $[Ca^{2+}]_i$ increases in primary CGN cultures. Experiments in CGN cell cultures assessed how the NO signaling pathway influences intracellular Ca^{2+} signaling to shield developing neurons from alcohol toxicity. Altering the time of alcohol exposure in relation to the addition of PKG activator, 8-Br-cGMP, established that PKG's reduction of alcohol toxicity depends on preventing alcohol-induced $[Ca^{2+}]_i$ elevations. For contrast, how brain-derived neurotrophic factor (BDNF), a protective agent that does not rely on PKG, affects alcohol-induced $[Ca^{2+}]_i$ elevations was also tested.

Understanding these interactions will advance the development of novel therapeutic strategies in preventing FASD by enabling better insight both into the intracellular pathways related to alcohol-induced neuronal death and into the neuroprotective mechanisms that counteract them.

CHAPTER II
ALCOHOL INITIATES NEURONAL DEATH BY ENHANCING
INTRACELLULAR CALCIUM SIGNALING THROUGH INOSITOL
TRISPHOSPHATE RECEPTOR-DEPENDENT PATHWAYS

Background and Significance

Alcohol abuse during the third trimester of pregnancy (during synaptogenesis) triggers severe loss in vulnerable neuronal populations of the developing brain (Konovalov *et al.* 1997). These resulting brain abnormalities culminate in serious behavioral and cognitive deficits that persist into adulthood (Hannigan & Armant 2000) and are identified clinically as FASD. Fetal alcohol syndrome, the most severe form of FASD, is the leading cause of mental retardation in the western world (Abel & Sokol 1987). As the severity of the brain damage is clinically debilitating, understanding the molecular mechanisms underlying alcohol's developmental neurotoxicity is a main goal of alcohol research.

To seek better understanding of the molecular mechanisms underlying alcohol-induced neuronal death and its relationship to Ca^{2+} signaling, we employed a cell culture model consisting of primary CGNs. CGN cultures represent a vulnerable neuronal population where clinically-relevant alcohol doses produce cell death similar to that seen in animal models (Pantazis *et al.* 1993). In rodents, the period of greatest alcohol susceptibility lasts from postnatal day (PD) 4 to PD 9 (Maier *et al.* 1997, Livy *et al.* 2003, Maier *et al.* 1999). Following this critical period, alcohol's toxicity to neuronal cells is reduced, implying the acquisition of a protective mechanism. For this reason, all CGN cultures in this study were derived from neonatal mice on postnatal day 5 to 7.

Alcohol has long been known to disrupt intracellular Ca^{2+} levels. Elevations in $[\text{Ca}^{2+}]_i$ were first observed indirectly via electrophysiological studies conducted in mouse and rat synaptosomes (Harris & Hood 1980), in invertebrate mollusks (Camacho-Nasi &

Treisman 1986, Oyama et al. 1986), in neoplastic PC12 cell cultures (Messing et al. 1986), and in *Xenopus* oocytes (Wafford et al. 1989). The later development of Ca^{2+} -sensitive fluorescent dyes allowed the direct measurement of intracellular Ca^{2+} levels (Grynkiewicz *et al.* 1985). Hence, alcohol exposure has been observed to acutely raise intracellular Ca^{2+} : *in vitro*, in rodent synaptosomes (Daniell et al. 1987, Rezazadeh et al. 1989) and neonatal rat astrocyte cultures (Holownia et al. 1997, Hirata et al. 2006), and *in vivo*, in mouse (Stachecki & Armant 1996) and chick embryos (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005).

Judging from alcohol's impact on downstream PLC products, DAG, IP_3 , and Ca^{2+} , these Ca^{2+} surges emanate from enhanced heterotrimeric $\text{G}_{i/o}$ protein activity (Garic-Stankovic et al. 2005) to stimulate PLC activity (Katsura et al. 1994, Machu et al. 1989, Natsuki & Yamaguchi 1996, Hoek et al. 1987). In general, ligand-binding to G protein-coupled receptors (GPCRs) initiates a conformational shift that alters the $\text{G}\alpha$ subunit's affinity for phosphonucleotides, causing GDP to be exchanged for GTP (Hamm & Gilchrist 1996). In turn, GTP binding results in the dissociation of $\text{G}\alpha$ from the $\text{G}\beta\gamma$ subunit, thus allowing each to interact with their respective downstream effectors. Interaction of $\text{G}\alpha_q$ or $\text{G}\beta\gamma$ with PLC promotes phosphoinositide turnover (McCudden *et al.* 2005, Drin & Scarlata 2007), thus boosting cytosolic IP_3 and DAG levels (Berridge & Irvine 1989). By generating IP_3 , alcohol triggers Ca^{2+} release via IP_3Rs located on the ER (Furuichi & Mikoshiba 1995, Ma et al. 2000). In gastric mucosal cell lines (Miller et al. 2001, Kokoska et al. 1999) and rat hepatocytes (Hoek et al. 1987, Reinlib et al. 1990a), alcohol elicited concomitant increases for both cytosolic IP_3 and Ca^{2+} , which is consistent with IP_3R activation and Ca^{2+} release from ER stores. In mouse and chick embryos, pharmacological inhibition of PLC, but not of PKC, prevented alcohol-dependent intracellular Ca^{2+} surges and subsequent cell death (Stachecki & Armant 1996, Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005). Since PKC is the

downstream target for DAG, this implied that increased IP₃ levels are responsible for alcohol-induced Ca²⁺ surges.

Notably, although these findings have been implicated in the mechanism of alcohol-related neuronal cell death, they have never before been described in primary mammalian neuronal cell cultures. In the following sets of experiments, we focused on the role that the IP₃R plays in both alcohol-induced disruption of [Ca²⁺]_i (Patterson *et al.* 2004) and neuronal death. We hypothesized that alcohol exposure of CGN cultures increases [Ca²⁺]_i by activating IP₃Rs, and that inhibition of the alcohol-induced rise in [Ca²⁺]_i reduces the neuronal death caused by alcohol in CGN cultures. Alcohol's induction of the PLC pathway and our hypotheses are summarized in Figure II-1. Since we observed that alcohol boosts intracellular Ca²⁺ within minutes, but cell death is apparent within hours; we also examined the temporal relationship between the elevation of [Ca²⁺]_i and alcohol-induced neuronal death.

Materials and Methods

Cell Culture

Primary cerebellar granule neuron (CGN) cultures were established from the cerebella of neonatal wild-type mice (5-7 days old) housed in The University of Iowa Animal Care Facility as previously described (Pantazis et al. 1995). Briefly, cerebella from each litter were excised, pooled, minced, and then trypsinized (0.125%) for dissociation and to avoid microglia contamination (Almeida & Medina 1998). Cells were dissociated by trituration in N-2 supplemented DMEM to produce a cell suspension. These procedures yield cell cultures comprised of 95% CGN (Dutton 1990). Greater detail is provided in chapter III.

For cell survival studies, cells were plated into poly-D-lysine (PDL, 50 mg/ml)-coated 96-well tissue culture plates at a density of 1.5 x 10⁶ cells/ml (300 µl/well).

Separate plates were used for alcohol-exposed and alcohol-free groups, since plates were placed in individual sealed containers to maintain alcohol concentration (see below).

For Ca^{2+} measurements, cells were plated onto PDL-coated (100 mg/ml) glass coverslips (25 mm in diameter), which were placed in the individual wells of a 6-well tissue culture plate. Cells were plated at a density of 1.0×10^6 cells/ml (2.0 ml/well). Prior to use, all CGN cultures were plated overnight at 37°C in humidified atmosphere containing 5% CO_2 / 95% air.

Pharmacological Treatments and Ethanol Exposure

CGN cultures were treated with one of the following agents according to the experimental paradigms as described in the following section: 1. PBS (vehicle control); 2. BAPTA acetoxymethyl ester (BAPTA/AM, Ca^{2+} chelator); 3. EGTA acetoxymethyl ester (EGTA/AM, Ca^{2+} chelator); 4. 2-aminoethoxydiphenyl borate [(2-APB, an IP_3R inhibitor that blocks IP_3 -dependent Ca^{2+} release from the ER)]; 5. xestospongin C (X-C, an IP_3R inhibitor); 6. dantrolene (an inhibitor of RyRs that blocks Ca^{2+} -induced Ca^{2+} release from the ER via RyRs); 7. U-73122 (a PLC inhibitor that block the formation of IP_3); 8. U-73343 (an inactive analog of PLC inhibitor U-73122); 9. FK-506 (calcineurin inhibitor); 10. KN-93 (CaMKii inhibitor); 11. KN-92 (an inactive analog of CaMKii inhibitor, KN-92); 12. Ru360 (an inhibitor of the MCU that blocks mitochondrial Ca^{2+} entry); 13. bongkreikic acid (an inhibitor of adenine nucleotide translocase (ANT) that prevents MPTP opening). All agents that were used in experiments are plasma membrane-permeable. As some agents (2-APB, X-C, BAPTA/AM, EGTA/AM, U-73343, U-73122, FK-506) required the solvent dimethyl sulfoxide (DMSO), all wells received identical DMSO exposure within each experiment.

CGN cultures were treated with pharmacological agents or vehicle control either thirty minutes prior to, simultaneously, or thirty minutes after initiating ethanol exposure. Ethanol (95%) was diluted in PBS and added (10 μl) to the culture medium to obtain final

concentrations of 200, 400 or 800 mg/dl (43, 87, or 174 mM). For simultaneous treatments, ethanol and pharmacological agents were prepared separately but mixed together prior to adding to the culture medium. To prevent alcohol loss by evaporation, the ethanol-exposed tray was placed in a sealed chamber containing an alcohol bath of equal concentration to the culture medium and 5% CO₂. Ethanol-free cultures were placed in containers with a water bath and 5% CO₂. For treatment paradigms that required agent administration thirty minutes after ethanol exposure, culture trays were briefly removed from their containers to receive pharmacological agents or a vehicle control before being resealed. Chambers were incubated at 37°C for 24 hours.

Cell Viability – Trypan Blue Exclusion

After 24 hours of ethanol exposure, CGN were suspended by trituration in 0.2% trypan blue. Viable cells were identified by dye exclusion and a phase contrast halo at the plasma membrane margin, a hallmark of viable cells when viewed with a phase-contrast microscope (Nikon Diaphot). Cell numbers for each well were estimated by counting cells on a hemacytometer (four counts per well) using a 20X objective. Cell numbers for each treatment condition within each litter were averaged.

Ca²⁺ Measurements – Fura-2 Fluorescence

Coverslips containing CGN (with cell density = 200,000 cells / cm²) were removed from media and washed (1X) with Hank's balanced salt solution (HBSS, 1 ml) to remove phenol red. Cultures were loaded with Ca²⁺-specific dye, Fura-2/AM (1.0 μM, 1 ml) for 30 minutes at 37°C then washed (1X, 1 ml) with HBSS to remove excess Fura-2/AM. Hydrolysis of intracellular Fura-2/AM to Fura-2 followed for 30 minutes at 37°C in HBSS (1 ml) in the presence of various agents as described in figure legends. Since various pharmacological agents required DMSO as a solvent, non-drug treatment control cultures received equivalent exposure to DMSO. Coverslips were transferred with fresh

identical media (400 μ l) to a temperature-controlled coverslip chamber (37°C) mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot). Cultures then received either the addition of 95% ethanol diluted in PBS (20 μ l) to the HBSS to obtain the final ethanol concentrations or a drug treatment as described in the figure legends. As was done in the survival studies for simultaneous treatments, ethanol and pharmacological agents were prepared separately but mixed together prior to adding to the culture medium.

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) was assessed by Fura-2 fluorescence ratio imaging on a digital imaging system [Photon Technology International (PTI) Deltascan-1]. A rotating chopper disk rapidly switched the light beam from a xenon arc lamp (75 W) between two monochrometers set at 340 nm and 380 nm, thus producing a monochromatic beam of ultraviolet (UV) light of alternating wavelengths. This UV beam was focused on CGN with a 40X oil-immersion objective (Nikon Fluor 40/1.30). Fluorescence output at 510 nm was captured with an intensified charge coupled device camera (PTI IC-200), digitized by a frame grabber board (Scion AG-5), and stored on computer.

Basal $[\text{Ca}^{2+}]_i$ was established for 15 seconds before ethanol or drug exposure. Real-time shifts in Fura-2 emission ratio fluorescence (Excitation: 340 / 380 nm), indicating changes in $[\text{Ca}^{2+}]_i$, were recorded before (basal) and during ethanol or drug-treatment exposures at 5 second intervals for the indicated durations. To compute average whole cell $[\text{Ca}^{2+}]_i$ from ratio image pairs, a mask was subsequently drawn around each cell from images captured at 340 nm excitation as previously described (Sharma *et al.* 1995). All cells within the microscope view field on each coverslip (an average of approximately 40 cells, ranging from 15 to 50 cells, per coverslip) were analyzed. To verify instrumentation, 10 μ M ionomycin was added at the end of the testing period, to elicit a Ca^{2+} response.

In each experiment, a minimum of three litters were used.

Statistical Analyses

In viability studies, statistical differences due to ethanol were determined by repeated measures ANOVA followed by Bonferroni-corrected pairwise comparisons. Ethanol-induced percent cell loss was calculated by comparing cell numbers in ethanol-treated conditions to respective non-ethanol treatment controls. Statistical differences in ethanol-induced cell loss were determined by one-way or two-way ANOVAs followed by Scheffé post-hoc tests ($p < 0.05$) as indicated.

Determinations of $[Ca^{2+}]_i$ from ratio image pairs were done by PTI ImageMaster software using the following equation (Grynkiewicz et al. 1985):

$$[Ca^{2+}]_i = \frac{S_{f2}}{S_{b2}} \times (K_D) \times \frac{(R - R_{min})}{(R_{max} - R)}$$

where R is the ratio of fura-2 fluorescence intensity attained at 340 nm over that at 380 nm; K_D is the Fura-2 dissociation constant (224 nM); R_{min} and R_{max} are the intensity ratios correspondingly achieved with maximally free (no calcium) and bound Fura-2 (saturating calcium); and S_{f2} and S_{b2} are the intensities reached at 380 nm excitation for solutions with maximally free and bound Fura-2, respectively. To compute average whole cell $[Ca^{2+}]_i$ from ratio image pairs, a mask was subsequently drawn around each cell from images captured at 340 nm excitation as previously described (Sharma et al. 1995). All cells within the microscope view field on each coverslip (an average of approximately 40 cells, ranging from 15 to 50 cells, per coverslip) were analyzed.

For calibration, the ratio and intensity values for maximally calcium-bound Fura-2 were obtained using 10 μ M ionomycin to generate ceiling $[Ca^{2+}]_i$ levels. Similarly, ratio and intensity values for maximally unbound Fura-2 were generated using 10 mM EGTA to chelate Ca^{2+} . Empty PDL-treated coverslips with HBSS were used to generate background values.

A basal $[Ca^{2+}]_i$ level was calculated for each cell, as the average $[Ca^{2+}]_i$ from the 15 seconds preceding drug or alcohol exposure. A peak $[Ca^{2+}]_i$ level was calculated from

the maximum $[Ca^{2+}]_i$ level attained following drug or alcohol addition during the sampling period indicated in the text (Sharma & Bhalla 1989). Analysis of $[Ca^{2+}]_i$ levels in this manner accounts for the variability in time between cells to respond to ethanol.

All statistical differences were determined using SPSS Statistics. Because of the changing design between experiments, specific statistical analyses are described within the text.

Results

Prior studies in CGN cultures derived from 9 to 10 day old litters showed that 24-hour alcohol exposure induced neuronal death, even with an alcohol concentration as low as 100 mg/dl (Pantazis et al. 1993). The experiment shown in Figure II-2 verified that work, demonstrating that 24-hour alcohol exposure induces a dose-dependent reduction in cell number of CGN cultures derived from litters 5 to 7 days old. The overall effect of alcohol on surviving cell numbers was significant as determined by repeated measures ANOVA [$F(3,10) = 15.02, p < 0.001$] and Bonferroni pairwise comparisons ($p < 0.001$). In brief, a 24-hour alcohol exposure at 200, 400, or 800 mg/dl significantly reduced cell numbers by 10%, 22%, or 32% respectively which demonstrates a dose-dependent relationship between the death of primary neurons in culture and alcohol. Alcohol has been established by others to reduce cell number either by inducing cell death (Olney *et al.* 2000) or by inhibiting cell proliferation (Liu *et al.* 1998). Since previous cell cycle analyses (Pantazis et al. 1993) established that cells in our CGN cultures do not proliferate, alcohol must be inducing neuronal death in this cell culture model.

Alcohol Elevates Intracellular Calcium

The experiment described in Figure II-3 reveals that alcohol induced a rapid rise in intracellular Ca^{2+} . Fura-2-loaded CGN cells on coverslips were pretreated with HBSS for 30 minutes prior to ethanol exposure (0, 200, 400, 800 mg/dl), and the resulting Fura-

2 fluorescence was measured for five minutes. Figure II-3A demonstrates a dose-dependent alcohol-induced increase in $[Ca^{2+}]_i$ as seen in the field-of-view from representative coverslips. With greater alcohol concentrations, more neurons in the lower panels (after ethanol exposure) exhibited increased $[Ca^{2+}]_i$ levels as indicated by color changes from blue-green (above) to yellow-red (below) in the pseudocolor 340/380 nm ratio images.

Summary time-course data in Figure II-3B highlights this effect, showing that greater alcohol concentrations produced both a more rapid rise and larger increase in $[Ca^{2+}]_i$. Addition of 800 mg/dl ethanol induced a rapid $[Ca^{2+}]_i$ surge, whereas 400 mg/dl initiated a more gradual rise, both attaining a mean of 65 nm above basal $[Ca^{2+}]_i$ levels (~65 nM) within 180 seconds. Ethanol exposure at 200 mg/dl increased $[Ca^{2+}]_i$ to 90 nM by 300 seconds. Alcohol-induced Ca^{2+} levels did not return to baseline during the five minute sample period. Longer recordings up to 30 minutes (data not shown) also show elevations in Ca^{2+} . Traces from 400 and 800 mg/dl alcohol doses plateaued at a higher level (120 nM), than that of 200 mg/dl (90 nM). Lastly, a small but significant increase in Fura-2 fluorescence was also observed with 0 mg/dl alcohol exposure over the time course of the experiment [paired t-test, $t(267) = -16.30$, $p < 0.001$].

Analysis of peak $[Ca^{2+}]_i$ levels attained in the five minutes following the addition of alcohol support these findings (Figure II-4A). Ethanol induced peak $[Ca^{2+}]_i$ levels of 88.9 ± 18.0 , 120.4 ± 16.9 , 171.5 ± 14.0 , and 243.6 ± 15.2 nM respectively for 0, 200, 400, and 800 mg/dl ethanol exposures and indicates a significant effect on peak $[Ca^{2+}]_i$ levels [one-way ANOVA, $F(3,1385) = 17.02$, $p < 0.001$], Scheffé post-hoc comparisons revealed that 400, 800 but not 200 mg/dl ethanol additions significantly produced higher elevations in $[Ca^{2+}]_i$ over that of PBS addition ($p < 0.005$). Analysis of this data grouped by repetitions of the experiment rather than by cells was also performed. For each day of experimentation (each litter), the average peak $[Ca^{2+}]_i$ of every ethanol concentration was calculated to provide the average peak $[Ca^{2+}]_i$ for every ethanol concentration on each

day of experimentation. A one-way ANOVA testing how ethanol concentration affected peak $[Ca^{2+}]_i$ levels agreed with the earlier analysis that used cells as samples, indicating an ethanol effect [$F(3,12) = 4.51, p < 0.05$].

Since the standard errors in Figure II-4A grew with greater alcohol concentrations, peak $[Ca^{2+}]_i$ levels for neurons in each alcohol treatment group were sorted into 20 nM bins and plotted in histograms (Figure II-4B) to visualize the distribution of the alcohol-induced $[Ca^{2+}]_i$ response (Hegarty *et al.* 1997). For cultures receiving 0, 200, or 400 mg/dl ethanol, the largest percentage of neurons in each panel attained peak $[Ca^{2+}]_i$ levels in the range of only 60 to 80 nM, whereas cultures administered 800 mg/dl ethanol, reached the range of 100 to 120 nM. The histograms also show that greater alcohol concentrations increased the range of peak $[Ca^{2+}]_i$ that neurons attained. With either 400 or 800 mg/dl alcohol concentrations, alcohol-sensitive neurons reacted over a large range. This is consistent with the hypothesis that alcohol-vulnerable populations within the developing CNS exist (Pantazis *et al.* 1993). Furthermore these data suggest that 800 mg/dl alcohol affects a larger population of neurons that are normally insensitive to alcohol. In conclusion, alcohol produced a wide Ca^{2+} response that as a population is seen as rapid and dose-dependent elevations in intracellular $[Ca^{2+}]_i$.

Alcohol Toxicity Depends on a Rise of Intracellular

Calcium

Since alcohol influences $[Ca^{2+}]_i$ levels, we examined whether membrane-permeable Ca^{2+} chelators (BAPTA/AM and EGTA/AM) could ameliorate alcohol-induced neuronal death in CGN cultures. Thirty minutes prior to initiating alcohol (400 mg/dl) exposure, CGN cultures were treated with increasing doses of either BAPTA/AM or EGTA/AM. After a 24-hour alcohol exposure, viable cell numbers were determined on a hemacytometer by trypan blue exclusion. Without chelator pretreatment, alcohol

exposure produced 17.9 ± 2.3 to $18.0 \pm 2.1\%$ neuronal loss in CGN cultures (Figure II-5). Pretreatment with the higher doses of either BAPTA/AM [$F(3,39) = 5.80, p = 0.005$] or EGTA/AM [$F(3,42) = 13.50, p < 0.001$] provided significant neuroprotection reducing alcohol-induced cell death to below 5%. Although negative cell loss (an increase in cell number) appears at the higher doses of EGTA, this is a natural result of experimental variability.

In light of the neuroprotection afforded by Ca^{2+} chelation, we tested whether the highest dose of BAPTA/AM ($1.0 \mu\text{M}$) used in Figure II-5 negates the observed alcohol-induced rise in $[\text{Ca}^{2+}]_i$ seen in Figure II-3. Fura-2-loaded CGN cultures were pretreated for 30 minutes with BAPTA/AM or with a comparable control containing an identical amount of DMSO. As before, fura-2 ratio fluorescence was measured from 15 seconds before exposure to ethanol (400 mg/dl) or PBS to 300 seconds afterwards and the resulting $[\text{Ca}^{2+}]_i$ levels were calculated. Shown in Figure II-6A, BAPTA/AM pretreatment completely eliminated the alcohol-induced rise in $[\text{Ca}^{2+}]_i$. In the absence of BAPTA/AM pretreatment, $[\text{Ca}^{2+}]_i$ levels in alcohol-treated cells surged, reaching maximum levels with 120 seconds of exposure. In comparison, CGN cultures pretreated with BAPTA/AM showed no rise in $[\text{Ca}^{2+}]_i$ like the cultures that receive no alcohol.

Analysis of the peak $[\text{Ca}^{2+}]_i$ levels [2 x 2 two-way ANOVA, $F(3,1279) = 26.53, p < 0.001$] attained in the five minutes following the initiation of alcohol exposure (Figure II-6B) also revealed that BAPTA/AM pretreatment affected the alcohol-induced $[\text{Ca}^{2+}]_i$ elevations [$F(1,1279) = 14.07, p < 0.001$]. In the absence of BAPTA/AM, ethanol (400 mg/dl) elevated peak $[\text{Ca}^{2+}]_i$ levels to $241.3 \pm 11.4 \text{ nM}$, as compared to $153.8 \pm 11.8 \text{ nM}$ reached for no alcohol exposure [$F(1,1279) = 10.84, p = 0.001$]. CGN cultures pretreated with BAPTA/AM realized lower peak $[\text{Ca}^{2+}]_i$ levels (107.6 ± 13.4 and $114.0 \pm 12.5 \text{ nM}$ for 0 and 400 mg/dl ethanol, respectively) [$F(1,1279) = 49.60, p < 0.001$]. As expected, the CGN cultures pretreated with the calcium chelator manifested lower basal $[\text{Ca}^{2+}]_i$ levels [two-sample t-test, $t(1176.77) = -5.890, p < 0.001$].

IP₃R-Dependent Ca²⁺ Release Mediates Alcohol-Induced

Cell Death

Although these results suggest that $[Ca^{2+}]_i$ levels play a key role in alcohol-induced cell death, they do not identify the source of the higher $[Ca^{2+}]_i$ levels observed following alcohol exposure to CGN cultures. Two likely Ca^{2+} sources are: 1) intracellular stores including the ER; and 2) Ca^{2+} uptake from the culture media. Since PLC-stimulated IP₃R signaling represents a major method of intracellular Ca^{2+} mobilization from ER stores, we examined how IP₃R antagonism affected alcohol toxicity. Figure II-7 shows that IP₃R blockade from 2-APB [$F(3, 23) = 14.105, p < 0.001$] and X-C [two-sample t-test, $t(16) = 15.50, p < 0.001$] pretreatment abolished alcohol-induced cell death, mimicking the neuroprotective effect of the cell-permeable Ca^{2+} chelators. X-C pretreatment reduced mean ethanol-induced cell death from $23.7 \pm 8.9\%$ to $1.0 \pm 7.4\%$. Pretreatment with 0.3, 1.3 and 5.0 μM 2-APB diminished cell death from $21.5 \pm 3.1\%$ to 14.0 ± 2.9 , 5.4 ± 3.0 , and $-0.9 \pm 1.1\%$, respectively. A Scheffé post-hoc test indicated that neuroprotection from 2-APB dose was dose-dependent ($p < 0.05$).

Since RyRs on ER membranes signify another avenue for ER Ca^{2+} efflux, we also tested how RyR inhibition by dantrolene influences alcohol-induced neuronal death. Thirty minutes before initiating a 24-hour alcohol exposure, CGN cultures were pretreated with either a membrane-permeable RyR inhibitor, dantrolene or an IP₃R inhibitor, 2-APB. Pretreatment with 5.0 μM 2-APB (Figure II-8) served as a neuroprotective control, completely eliminating the alcohol-induced cell death ($19.4 \pm 3.8\%$) observed without 2-APB pretreatment [two-sample t-test, $t(8) = 3.67, p = 0.006$]. In contrast, no discernable resistance was detected from RyR blockade, in that at all doses of dantrolene tested, cell death remained between $18.5 \pm 4.6\%$ and $21.4 \pm 5.1\%$ [$F(3,16) = 0.15, p = 0.927$].

Since IP₃R but not RyR antagonism prevented alcohol-induced cell death, we examined how IP₃R blockade affected the alcohol-induced Ca²⁺ surges. Pretreatment with 2-APB (5 μM) or DMSO (control) was followed by 400 mg/dl ethanol exposure in fura-2-loaded CGN cultures. Shown in Figure II-9A are photomicrographs that are representative of the data collected in this experiment. Prior to ethanol exposure (panels a - b), there are few neurons displaying high levels of [Ca²⁺]_i, being mostly blue-green with very few red-colored in the pseudocolor map. When the same neurons are viewed after initiating alcohol exposure, it is evident that the number of red-shaded neurons has increased substantially in the group receiving DMSO pretreatment (panel c). In contrast, the group pretreated with 2-APB had little or no increase in the numbers of red-shaded neurons at one minute (panel d).

Analysis of the peak [Ca²⁺]_i levels [2 x 2 two-way ANOVA, $F(3,1321) = 31.08$, $p < 0.001$] for each of these cell populations (Figure II-9B) yielded a main effect of alcohol exposure [$F(1,1279) = 10.84$, $p = 0.001$] and revealed an interaction between 2-APB pretreatment and alcohol exposure in that 2-APB pretreatment affected the alcohol-induced [Ca²⁺]_i elevations [$F(1,1321) = 41.91$, $p < 0.001$]. Without 2-APB, ethanol elevated peak [Ca²⁺]_i levels to 142.8±6.5 nM, as compared to 81.4±2.7 nM reached for no alcohol exposure. With 2-APB pretreatment, ethanol failed to increase peak [Ca²⁺]_i levels, being 106.3±4.7 nM and 106.4±4.1 nM for ethanol and no ethanol additions, respectively. No main effect of prior 2-APB treatment on peak [Ca²⁺]_i levels was observed. Overall, visual inspection of fura-2 response and the analysis of the peak [Ca²⁺]_i levels suggested that IP₃R inhibition by prior 2-APB exposure prevented an alcohol-increase in [Ca²⁺]_i. Collectively, these data suggest that alcohol initiates Ca²⁺ release via IP₃R activation.

Since PLC activation often leads to IP₃R stimulation, we tested how inhibition of PLC affects CGN survival following alcohol exposure. CGN cultures were treated with either a PLC inhibitor (U-73122) or its respective inactive analog (U-73343) prior to 24-

hour ethanol exposure (400 mg/dl). As shown in Figure II-10, U-73122 at the highest tested dose reduced alcohol-induced cell loss to $2.2 \pm 5.2\%$, whereas cell loss remained at between 20.7 ± 3.4 to $25.4 \pm 3.7\%$ with pretreatment by the inactive analog, U-73343. A one-way ANOVA supported this assessment in that U-73122 [$F(3,36) = 6.54, p = 0.001$] but not U-73343 [$F(3,36) = 0.23, p = 0.877$] reduced alcohol-induced cell death. Since PLC inhibition strongly protects CGN cultures from alcohol toxicity, it is likely that alcohol-induced neuronal death involves PLC.

Alcohol-Induced IP₃R-Dependent Ca²⁺ Release Originates from Intracellular Stores

To rule out extracellular Ca²⁺ as the source of the Ca²⁺ surge, we investigated how removal of Ca²⁺ from the culture media affected the alcohol-induced rise in [Ca²⁺]_i. Thirty minutes prior to alcohol exposure, CGN cultures were pretreated with 2-APB (5 μM), or HBSS containing an identical amount of DMSO. Just before imaging, cultures were transferred to HBSS media either containing 1.26 mM Ca²⁺ or without Ca²⁺. In the absence of 2-APB, ethanol raised [Ca²⁺]_i regardless of whether Ca²⁺ was present in the culture media (Figure II-11A). 2-APB, which blocks IP₃Rs on the ER membrane, eliminated the alcohol-induced rise of [Ca²⁺]_i. Analysis of the peak [Ca²⁺]_i levels (Figure II-11B) with a 2x2 two-way ANOVA supports this observation in that although 2-APB pretreatment reduced peak [Ca²⁺]_i from 217 ± 4.4 to 152.4 ± 5.5 nM [$F(1,966) = 83.9, p < 0.001$], the extracellular presence of calcium failed to affect [Ca²⁺]_i levels [$F(1,966) = 0.763, p = 0.383$]. When considered in tandem, this suggests the elevation in [Ca²⁺]_i from alcohol exposure at 400 mg/dl originates from an intracellular source instead of an extracellular one. It is of interest that calcium levels in all conditions continued to rise throughout the experiment (nine minutes).

Alcohol Induces Neuronal Death at the Initial Exposure

In our cell culture model of alcohol toxicity, alcohol was present for 24 hours before assessing cell death, although it is evident that alcohol elevates $[Ca^{2+}]_i$ within minutes of its addition. The pharmacological agents that prevent alcohol toxicity and block the $[Ca^{2+}]_i$ surge were also present during the entire length of the alcohol exposure. We therefore wondered if this initial $[Ca^{2+}]_i$ surge was linked to alcohol-induced cell death. Having established that pretreatment with calcium chelators and IP₃R antagonists block the alcohol-induced calcium elevations, we manipulated temporally the addition of these protective agents in relation to the alcohol exposure (Figures II-12 and II-13). In this way, we assessed whether the prevention of the calcium elevation or the continued presence of the protective agents were important deterrence factors in the amelioration of alcohol's toxicity.

Indeed, we observed that calcium chelation and IP₃R inhibition must precede alcohol exposure to impede alcohol toxicity (Figure II-12). When added 30 minutes prior to alcohol exposure, BAPTA/AM and 2-APB reduced alcohol toxicity from 22.5±2.3 to 2.9±0.62% and 21.4±2.5 to -2.3±2.9%, respectively. When added simultaneously with or after alcohol exposure, cell death remained at between 17.3±2.5 to 22.5±2.3%. For calcium chelation, alcohol-induced cell death was subjected to a 2x3 two-way ANOVA with BAPTA/AM treatment and alcohol exposure in relation to BAPTA/AM treatment as independent measures. As evident in Figure II-12, BAPTA/AM treatment and alcohol exposure in relation to BAPTA/AM treatment yielded a significant interaction [$F(2,30) = 10.9, p < 0.001$]. Similarly, a 2x3 two-way ANOVA for IP₃R antagonism and treatment time in relation to alcohol exposure also generated an interaction effect [$F(2,30) = 12.6, p < 0.001$], signifying that 2-APB was only effective in diminishing cell death when it preceded alcohol exposure. In conclusion the addition of these protective agents must precede alcohol exposure to be neuroprotective.

As indicated by Figures II-5, II-6, II-7, and II-9, pretreatment with BAPTA/AM or 2-APB eliminated alcohol-induced Ca^{2+} elevations and cell death. Considering that concomitant addition of BAPTA/AM and 2-APB with alcohol failed to ameliorate cell death, it is likely that the simultaneous addition of these agents would also not affect alcohol-induced Ca^{2+} elevations. To investigate this hypothesis, whether simultaneous addition of these agents with alcohol still permitted an $[\text{Ca}^{2+}]_i$ surge, the peak $[\text{Ca}^{2+}]_i$ levels attained were measured in Fura-2-treated CGN cultures under the conditions described in Figure II-13. CGN cultures were treated with BAPTA/AM (1.0 μM) or 2-APB (5.0 μM) either 30 minutes before, or simultaneously with ethanol (400 mg/dl). DMSO additions served as the vehicle control. Prior BAPTA/AM and 2-APB additions reduced the mean peak $[\text{Ca}^{2+}]_i$ attained from 114.6 ± 5.6 and 127.29 ± 7.6 nM to 59.9 ± 2.1 and 77.0 ± 3.3 nM, respectively. Peak $[\text{Ca}^{2+}]_i$ levels remained at 146.6 ± 10.1 nM with concurrent 2-APB additions with alcohol (Figure II-13B). Concurrent BAPTA/AM addition (Figure II-13A) resulted in peak $[\text{Ca}^{2+}]_i$ levels of 88.1 ± 2.7 nM, which is both less than that attained by DMSO pretreatment (114.6 ± 5.6 nM) and greater than that attained (59.9 ± 2.1 nM) by prior BAPTA/AM addition (Scheffé, $p < 0.001$). Although this may indicate that BAPTA/AM chelates Ca^{2+} with enough efficiency to affect the peak $[\text{Ca}^{2+}]_i$ levels attained during the sampling period, the binding kinetics of BAPTA/AM to Ca^{2+} are not a focus of this experiment. Neither peak $[\text{Ca}^{2+}]_i$ reached by concomitant addition of BAPTA/AM or 2-APB differed significantly from that of concomitant DMSO additions with alcohol (Scheffé, $p = 0.760$; $p = 0.963$).

Downstream Effectors of Calcium Mediate Alcohol-Induced Neuronal Death

As a signaling molecule, calcium has multiple downstream effectors. Having determined that alcohol elevates intracellular calcium to induce cell death, logically the next step was to identify which pathways calcium stimulates to initiate cell death. Since

calcineurin and CaMKii are common calcium-activated cytosolic enzymes, we tested if pharmacological blockade of either calcineurin or CaMKii protects against alcohol toxicity. Before overnight ethanol exposure (400 mg/dl), CGN cultures were pretreated with calcineurin inhibitor, FK-506 (Figure II-14A), or with CaMKii inhibitor, KN-93, or its inactive analog, KN-92 (Figure II-14B). Addition at higher concentrations for both agents was neuroprotective. At higher doses, FK-506 protected CGN cultures from alcohol toxicity [one-way ANOVA, $F(5,48) = 6.31, p < 0.001$] so that at 500 nM, FK-506 reduced alcohol-induced cell loss from 15.9 ± 1.7 to $-1.8 \pm 2.9\%$ (Scheffé, $p = 0.024$). KN-93 addition diminished alcohol-induced cell death [one-way ANOVA, $F(3,44) = 13.2, p < 0.001$] having decreased cell death from 21.7 ± 1.3 to 2.3 ± 1.7 at 1.0 μM . KN-92 addition at 1.0 μM also displayed a slight protective effect reducing alcohol-induced neuronal death to $13.6 \pm 2.9\%$ [$t(18) = 2.50, p = 0.022$]. However, increasing its concentration failed to increase KN-92's protective effect which remained in the range of 13.6 ± 2.9 and $14.69 \pm 2.6\%$ [one-way ANOVA, $F(3,36) = 2.70, p = 0.060$]. This indicates that the mechanism that achieves this slight protective effect quickly becomes saturated.

Elevations in mitochondrial calcium are linked to formation of MPTP, which when open leads to apoptotic cell death (Baumgartner *et al.* 2009). Inhibition of adenine nucleotide translocase (ANT), a MPTP component, prevents MPTP opening, thereby inhibiting apoptosis (Halestrap & Brenner 2003). To enter the mitochondria and influence MPTP conductance, calcium must enter through the MCU (Putney & Thomas 2006). For these reasons, it seemed sensible that they may be involved downstream of calcium in alcohol-induced cell death.

Thirty minutes before overnight alcohol exposure, we treated CGN cultures with bongkreikic acid (Henderson & Lardy 1970), an inhibitor of the ANT, or with Ru360 (Zazueta *et al.* 1999), an inhibitor of the MCU, and assessed their effects on alcohol-induced cell death. Increasing the concentration of either agent attenuated alcohol-induced cell death [bongkreikic acid, $F(3,12) = 11.13, p = 0.001$; Ru360, $F(6,28) = 12.10,$

$p < 0.001$]. 25.0 μM bongkreikic acid reduced cell loss from 16.3 ± 0.7 to $2.4 \pm 2.6\%$; 1.0 nM Ru360, from $16.7 \pm 1.3\%$ to $-1.8 \pm 2.5\%$.

Discussion

The movement of intracellular Ca^{2+} across cellular compartments is carefully regulated both spatially and temporally. Each compartment also maintains specific Ca^{2+} concentrations that involve steep concentration gradients across membranes as is evident in the endoplasmic reticulum and the mitochondria. Since calcium is an extremely versatile second messenger implicated in a wide array of cellular processes, disruption of the carefully maintained calcium homeostasis often results in pathological signals that cumulate in apoptosis. In the context of FASD, the present study linked alcohol-induced neuronal death to Ca^{2+} elevations that emanate from intracellular stores and are PLC-IP₃R dependent. Most importantly, it demonstrated that this Ca^{2+} surge, which occurs within minutes of alcohol exposure, is an essential key event in alcohol-induced neuronal death.

Alcohol's Effects on Cell Death and $[\text{Ca}^{2+}]_i$ are

Dose-Dependent

The experiment described in Figure II-2 indicates that 24-hour alcohol exposure produces a concentration-dependent loss in CGN cultures derived from 5 to 7 day old mice. This is consistent with previous findings in CGN cultures derived from 9 to 10 day neonatal rats, where alcohol also produced a dose-dependent neuronal death (Pantazis et al. 1993). Although ethanol at 200 mg/dl produced statistically significant cell loss, we used 400 mg/dl for most of our experiments, since alcohol-related effects can be more easily seen using fewer animals at the higher dose. One may argue that a BAC of 400 mg/dl in most individuals achieves respiratory depression and death, and consequently cannot be considered a physiological dose. However, chronic alcohol abusers easily

attain this BAC with only the appearance of slight intoxication or sobriety due to alcohol's upregulation of the MEOS (Lieber 1999, Lieber & DeCarli 1968).

Similar to the concentration-dependent CGN loss, we observed that acute alcohol exposure produced a dose-dependent elevation in intracellular calcium (Figures II-3 to 4). This is not surprising considering that comparable alcohol-induced enhancements of calcium signaling have been reported in numerous previous studies (Harris & Hood 1980, Camacho-Nasi & Treistman 1986, Oyama et al. 1986, Messing et al. 1986, Wafford et al. 1989, Daniell et al. 1987, Rezazadeh et al. 1989, Holownia et al. 1997, Hirata et al. 2006, Stachecki & Armant 1996, Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005). To our knowledge, despite alcohol's effect on $[Ca^{2+}]_i$ being well-known in the literature, this is the first evidence in CGN cultures of a dose-dependent effect from acute alcohol exposure on $[Ca^{2+}]_i$. A dose-dependent alcohol-induced Ca^{2+} response has been observed previously in chick embryos (Garic-Stankovic et al. 2006). The sudden surge in $[Ca^{2+}]_i$ observed with the 800 mg/dl ethanol addition starkly contrasted with the gradual elevations produced from 200 and 400 mg/dl ethanol additions, which indicates that 800 mg/dl ethanol concentration may result from fundamentally different effects physiologically. The histograms show that greater alcohol concentrations increased the range of peak $[Ca^{2+}]_i$ that neurons attained which is consistent with the hypothesis that alcohol-vulnerable populations within the developing CNS exist (Pantazis et al. 1993).

Alcohol-Induced $[Ca^{2+}]_i$ Elevations Originate from IP_3 -Dependent Intracellular Stores

Two observations support the hypothesis that alcohol increases $[Ca^{2+}]_i$ by activating IP_3 Rs to release Ca^{2+} from intracellular stores. First, alcohol-induced increases in $[Ca^{2+}]_i$ were still observed from CGN cultures in Ca^{2+} -free medium (Figure II-11), which suggests an intracellular source for Ca^{2+} elevation. Second, blocking IP_3 Rs with 2-APB prevented alcohol-induced increases in $[Ca^{2+}]_i$ (Figures II-9 and II-11). It is likely

that these Ca^{2+} elevations originate from IP_3R -dependent ER stores since the IP_3Rs found in the cerebellum are located mainly on ER membranes (Taylor et al. 1999).

Conversely, IP_3Rs have also been detected on nuclear membranes in cultured rat hippocampal neurons (Sato *et al.* 2005) and in *Xenopus* oocytes (Stehno-Bittel *et al.* 1995), which alludes to the possibility of an additional nuclear source for the alcohol-induced Ca^{2+} elevations. Indeed, the cell nucleus is the third largest intracellular calcium store after the ER and the mitochondria. However, in the isolated nuclei of cerebellar Purkinje and granule cells, only Purkinje cell nuclei showed any IP_3 sensitivity indicating that CGN nuclei lack IP_3Rs (Marchenko & Thomas 2006). Although not tested here, this evidence suggests that alcohol-induced calcium elevations more likely emanate from the ER rather than the nucleus in our CGN cultures.

Another caveat is that these two experiments tested alcohol exposure only at 400 mg/dl. Therefore, they do not address the possibility that $[\text{Ca}^{2+}]_i$ elevations induced by higher alcohol concentrations may involve recruitment of additional signaling mechanisms or an extracellular source.

Alcohol-Induced $[\text{Ca}^{2+}]_i$ Elevations are Neurotoxic

Prevention of the alcohol-induced $[\text{Ca}^{2+}]_i$ increase in CGN cultures by Ca^{2+} chelation (Figure II-6) or IP_3R antagonism (Figures II-9 and II-11) eliminated alcohol-induced neuronal death (Figures II-5 and II-7), which indicates that alcohol's elevation of $[\text{Ca}^{2+}]_i$ is neurotoxic. These *in vitro* findings are consistent with *in vivo* evidence that describes BAPTA/AM abolishing both alcohol-induced neuronal death and calcium elevations in chick embryo neural crest (Debelak-Kragtorp et al. 2003). Although alcohol and the protective agents that prevent the $[\text{Ca}^{2+}]_i$ effect are present for 24 hours in our cultures before assessing cell loss, our data indicate that alcohol's initiation of cell death is an early event. Addition here of the Ca^{2+} chelators or IP_3R antagonists simultaneously or 30 minutes after alcohol failed to protect against alcohol

toxicity (Figure II-12). Concurrent addition with alcohol also failed to block the alcohol-induced rise in $[Ca^{2+}]_i$ (Figure II-13). This may indicate that with simultaneous addition, neither 2- BAPTA/AM nor APB have had time to properly chelate $[Ca^{2+}]_i$ or inhibit the IP_3R , thus allowing the alcohol-induced Ca^{2+} elevations. These results undeniably link alcohol's effect on $[Ca^{2+}]_i$ to the neuronal death which alcohol causes; suppressing alcohol's $[Ca^{2+}]_i$ effect spares the neurons.

However, it should be noted that not all Ca^{2+} elevations are toxic. In CGN cultures, NMDA elevates $[Ca^{2+}]_i$ to 175 nM and provides a trophic effect (Bhave 1997, Contestabile 2002). In fact, NMDA-mediated Ca^{2+} influx from extracellular space via NMDA receptors is neuroprotective against alcohol toxicity (West & Dai 1998). At the other end of the spectrum, the wasp venom toxin, mastoparan, raises $[Ca^{2+}]_i$ to 1700 nM in CGN cultures and induces neuronal death (Lin *et al.* 1997). Alcohol in our experiments increased peak $[Ca^{2+}]_i$ levels to between 175 and 250 nM, which are similar to $[Ca^{2+}]_i$ elevations seen with apoptotic doses of carbachol in CGN cultures (Yan *et al.* 1995). This two-fold increase is consistent with observed alcohol-induced values in the chick neural crest (Debelak-Kragtorp *et al.* 2003, Garic-Stankovic *et al.* 2005) and in cultured astrocytes (Hirata *et al.* 2006). Although comparable to the carbochol-induced elevations, this does not indicate that a fixed toxicity threshold for $[Ca^{2+}]_i$ exists between 175 and 250 nM. For example, even though CGN cells survive the apoptotic stimulus of serum withdrawal when cultured with high potassium concentrations (25 mM), they reach a $[Ca^{2+}]_i$ of 250 nM (Zhong *et al.* 2004). It is more likely that Ca^{2+} interact with multiple cellular processes that influence cell death, which may account for the innate vulnerability or resistance of cell populations to alcohol. It is important to remember that the local subcellular $[Ca^{2+}]_i$ are often greater at where Ca^{2+} enters the cytosol than in the entire cell and these microdomain may influence cell death (Rizzuto & Pozzan 2006). For example, neuroprotective NMDA induces Ca^{2+} elevations at the cell surface (Pantazis

et al. 1995) whereas many apoptotic stimuli result in local elevations at ER-mitochondria junctions (Rizzuto & Pozzan 2006).

Alcohol-Induced Cell Neuronal Death Depends on the PLC-IP₃R Pathway

As mentioned, PLC activity normally increases intracellular IP₃ levels which then activate IP₃Rs on ER membranes to release calcium into cytosol. Overall, this study achieved the important goal of demonstrating in mammalian neuronal cell cultures that PLC and IP₃R inhibition prevents alcohol-induced cell death and that IP₃R inhibition prevents the alcohol's elevation of calcium. This finding is similar to evidence from avian embryos showing that PLC or IP₃R inhibition prevented both alcohol-induced apoptosis and the calcium elevations (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005) and from other non-neural cell culture models that demonstrated increased PLC activity from alcohol exposure (Katsura et al. 1994, Machu et al. 1989, Natsuki & Yamaguchi 1996, Hoek et al. 1987, Miller et al. 2001, Kokoska et al. 1999, Reinlib et al. 1990). In mouse embryos, PLC but not PKC inhibition, prevented alcohol-dependent Ca²⁺ release from intracellular ER stores (Stachecki & Armant 1996), which indicates IP₃R involvement. In gastric mucosal cells (Miller et al. 2001, Kokoska et al. 1999) and rat hepatocytes (Hoek et al. 1987, Reinlib et al. 1990), alcohol also generated concomitant increases for both cytosolic IP₃ and Ca²⁺, which is consistent with IP₃ activation of the IP₃R.

In this study the involvement of the PLC-IP₃R pathway alcohol toxicity was indicated by the following observations: PLC inhibitor, U-73122, and IP₃R antagonists, 2-APB and X-C, reduced alcohol-induced neuronal death (Figures II-7 and II-10); alcohol-induced [Ca²⁺]_i elevations were suppressed by 2-APB pretreatment (Figure II-9). The experiments involving U-73122 are preliminary. Demonstrating that PLC inhibition

prevents alcohol-induced $[Ca^{2+}]_i$ elevations would solidify the hypothesis that alcohol requires the PLC-IP₃R pathway to produce toxic elevations in $[Ca^{2+}]_i$. Although not tested here, PLC inhibition in chick embryos abolished alcohol-induced calcium elevations (Debelak-Kragtorp et al. 2003). Alcohol-induced Ca^{2+} elevations originated from the intracellular stores (Figure II-11).

However, the observation made in chick embryos that IP₃R inhibition (via X-C) by itself promotes cell death without alcohol present (Garic-Stankovic et al. 2005), was absent in our cultures at the concentrations that we used (data not shown). This discrepancy may have resulted from our electing to use a simpler *in vitro* model to study alcohol toxicity, as *in vivo* neuronal survival during brain development relies on multiple other stimuli subject to interference from X-C. For example (Garic-Stankovic et al. 2005), X-C administration would interfere with the induction of neuronal differentiation by brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) in rat embryonic brain cells, which requires PLC-mediated IP₃ release (Widmer et al. 1993, Widmer et al. 1992).

Lastly, RyRs represent another route for calcium to exit the ER. In a pilot study, blocking RyRs failed to shield neurons from alcohol toxicity (Figure II-8). Although this preliminary experiment may indicate that alcohol toxicity relies solely on IP₃R activation, measuring $[Ca^{2+}]_i$ during RyR inhibition would allow the introduction of several important experimental controls. For instance, demonstrating that dantrolene can prevent RyR agonist-induced Ca^{2+} elevations would verify that RyR are effectively being inhibited. In addition, demonstrating that alcohol-induced Ca^{2+} elevations still occur in the presence of RyR inhibition would support the hypothesis that the alcohol-induced Ca^{2+} surges are toxic.

*Alcohol Activates Multiple Downstream Calcium Effectors
to Promote Cell Death*

Alcohol exposure within seconds produced modest but lethal increases in $[Ca^{2+}]_i$ which remained elevated for the entire examination period (usually 10 minutes). This observation begs the question of how such an early event, the $[Ca^{2+}]_i$ elevation, precipitates the cell death observed hours later. The present study sampled a small selection of apoptotic interactants, testing how the inhibition of calcineurin and CaMKii in the cytosol and the MCU and MPTP in the mitochondria affected alcohol-induced neuronal death.

As seen in Figure II-14, calcineurin inhibition by FK-506 protected CGN cultures from alcohol toxicity. IP_3R -mediated increases in cytosolic Ca^{2+} have been observed in neurons to activate calcineurin (Lossi *et al.* 2009) and subsequently induce apoptosis by two mitochondrial (intrinsic) apoptotic mechanisms. Calcineurin influences the subcellular locale and activity of BCL-2 (including BAD and BAX) family proteins to promote apoptosis (Wang *et al.* 1999, Shou *et al.* 2004). BAD's dephosphorylation by calcineurin initiates an apoptotic signaling cascade where BAD translocates to the mitochondria, the MPTP opens, and proapoptotic signaling molecules enter the cytosol from the mitochondria (Springer *et al.* 2000, Wang *et al.* 1999).

In the second mechanism, calcineurin dephosphorylates and activates glycogen synthase kinase-3 (GSK-3) (Kim *et al.* 2009), which is involved in the protein synthesis of proapoptotic factors (Takadera & Ohyashiki 2004) and promotes intrinsic apoptotic signaling cascades (Beurel & Jope 2006). Of interest to prenatal alcohol toxicity, GSK-3 inhibition shielded rat CGN cultures against alcohol exposure (de la Monte & Wands 2002, Luo 2009), whereas GSK-3 overexpression increases alcohol sensitivity in a neuroblastoma cell line (Liu *et al.* 2009). Prenatal alcohol exposure also enhanced GSK-3 activity, as determined by increased phosphorylation of tau, a GSK-3 β substrate (Chen *et al.* 2009, Luo 2009). One substrate of GSK-3, the antiapoptotic β -catenin, becomes

rapidly degraded by proteosomes when phosphorylated (Hino *et al.* 2005). Alcohol exposure of chick embryos has been recently observed to deplete neural crest nuclear β -catenin, an important trophic factor for neural crest survival (Garic-Stankovic *et al.* 2010).

Inhibition of CaMKii by KN-93 also diminished alcohol-induced neuronal death in our CGN cultures (Figure II-14). Transient increases in cytosolic Ca^{2+} obviously lead to sustained activation of CaMKii (Colbran 1992). In death receptor (extrinsic) apoptotic pathways, CaMKii is an active signaling molecule. CaMKii, in numerous cell culture models, induces the c-Jun N-terminal kinase apoptotic pathway via apoptosis signal-regulating kinase 1 and consequently upregulates the proapoptotic Fas (Kohn & Moon 2005, Timmins *et al.* 2009, Takeda *et al.* 2004, Matsukawa *et al.* 2004). CaMKii also mobilizes intrinsic apoptotic mechanisms in that CaMKii increases mitochondrial Ca^{2+} , causes cytochrome *c* release, and dissipates the mitochondrial membrane potential (Timmins *et al.* 2009).

Wnt signaling, which affects apoptosis and cell fate during development, also influences CaMKii activity (Li *et al.* 2006, Kuhl 2004, Kohn & Moon 2005). Since β -catenin is a central mediator, Wnt signaling is typically classified into β -catenin-dependent and -independent pathways. Wnt ligands that promote β -catenin alter cell proliferation and fate to increase body dorsalization and diminish ventral morphology (Larabell *et al.* 1997). The Wnt/ Ca^{2+} signaling cascade, a β -catenin-independent pathway involving CaMKii, has the opposite effect. CaMKii inactivation promotes embryo dorsalization (Kühl *et al.* 2000), whereas constitutively active CaMKii rescues embryos from hyperdorsalization (Westfall *et al.* 2003a). Similarly, IP_3R antagonism by X-C generates hyperdorsalization like that observed in *Xenopus* and zebrafish embryos with deficient Wnt/ Ca^{2+} signaling (Westfall *et al.* 2003a, Westfall *et al.* 2003b, Kume *et al.* 1997). Although the mechanism has yet to be established, increased CaMKii activity accompanies depletion of nuclear β -catenin, whereas diminished CaMKii activity results

in higher β -catenin levels (Liang *et al.* 2007). Interestingly, CaMKii but not calcineurin inhibition has been recently observed to prevent nuclear β -catenin depletion from alcohol exposure (Garic-Stankovic *et al.* 2010). These findings suggest that alcohol exposure may exacerbate the normal neuronal loss that occurs during brain development.

Ru360 inhibition of the MCU, through which Ca^{2+} enters mitochondria, also spared our CGN cultures from alcohol toxicity (Figure II-15). The close proximity of the IP₃Rs on ER membranes to mitochondria suggests that IP₃R-mediated Ca^{2+} signals propagate directly into the mitochondria (Otsu *et al.* 1990, Satoh *et al.* 1990). Studies visualizing Ca^{2+} uptake by mitochondria-targeted Ca^{2+} -sensitive compounds confirm that Ca^{2+} uptake quickly follows IP₃-induced Ca^{2+} release from the ER (Hajnoczky *et al.* 2000). Since inhibiting the MCU rescued our CGN cultures from alcohol toxicity, mitochondrial Ca^{2+} entry under these conditions must precipitate cell death.

Elevations in mitochondrial Ca^{2+} can facilitate the opening of the MPTP (Bernardi 1999) leading to mitochondrial rupture and the subsequent release of mitochondrial factors [cytochrome *c*, apoptosis-inducing factor (AIF), and Diablo/Smac] into the cytosol where they act as caspase cofactors and potentiate apoptosis (Kim *et al.* 2005). Indeed, IP₃-linked mitochondrial Ca^{2+} uptake has been observed to drive MPTP opening and cytosolic cytochrome *c* release (Szalai *et al.* 1999). It follows that by restricting mitochondrial Ca^{2+} uptake, MPTP opening would also be inhibited. Translocation of BAD to the mitochondria also sensitizes the MPTP to Ca^{2+} (Narita *et al.* 1998, Pacher & Hajnoczky 2001) signifying that calcineurin and mitochondrial Ca^{2+} uptake-mediated apoptotic mechanisms can act synergistically.

In our CGN cultures, MPTP inhibition, by bongkreik acid, eliminated alcohol-induced neuronal death (Figure II-15). This finding is consistent with other experimental evidence where alcohol potentiates MPTP opening. MPTP inhibitor, cyclosporin A, prevents MPTP opening after alcohol exposure, as evidenced by a decrease in mitochondrial depolarization, mitochondrial swelling, and Ca^{2+} and cytochrome *c* release

(Pacher & Hajnoczky 2001, Pastorino et al. 1999, Higuchi *et al.* 2001, Ramachandran et al. 2001). Alcohol and other apoptotic stimuli also direct cytosolic BAX to the mitochondria (Wolter et al. 1997, Adachi et al. 2004) to interact with MPTP, sensitizing it (Pastorino et al. 1999, Ramachandran et al. 2001). BAX contributes to a number of processes that destabilizes the mitochondrial membrane and includes Smac/Diablo (Zhou *et al.* 2005) and cytochrome *c* release (Korsmeyer et al. 2000). This in turn leads to caspase activation, AIF cleavage in neurons, and apoptosis (Jurgensmeier et al. 1998)

One important consideration is that the neuroprotection afforded by restricting mitochondrial Ca^{2+} uptake and inhibiting the MPTP may be generic; restricting mitochondrial Ca^{2+} uptake can inhibit the MPTP, which prevents the cell death induced by a multitude of apoptotic insults. Cell death depends on the extent of MPTP opening (Honda & Ping 2006). If the extent of MPTP formation is small, the cell may recover. If MPTP opening occurs to a great degree, the cell will undergo apoptosis.

Although implicated here in alcohol neurodevelopmental toxicity, Ca^{2+} uptake is essential for normal mitochondrial functioning (Duchen 2000); Ca^{2+} is required for ATP production by the dehydrogenases of Krebs's cycle (McCormack *et al.* 1990). In addition, mitochondria in excitable cells modulate cytosolic Ca^{2+} signals by sequestering cytosolic Ca^{2+} (Werth & Thayer 1994, Wang & Thayer 1996, Friel & Tsien 1994, Babcock *et al.* 1997, David *et al.* 1998), hence accelerating the recovery of voltage gated Ca^{2+} channels (Duchen 2000). In turn, this facilitates post-tetanic potentiation of synaptic transmission, and synaptic vesicle fusion (Tang & Zucker 1997, David et al. 1998). Mitochondrial Ca^{2+} uptake serves a physiological function. However, as discussed earlier, it can become pathological and precipitate cell death under the correct circumstances.

Conclusions and Future Directions

In summary, we characterized the alcohol-induced ER- Ca^{2+} release that occurs through IP_3Rs using primary CGN cultures. Our results suggest that alcohol produces a

dose-dependent increase in $[Ca^{2+}]_i$ and cell loss which are due to abnormal Ca^{2+} release from intracellular stores rather than Ca^{2+} influx from extracellular space. Inhibitors of ER- Ca^{2+} release and Ca^{2+} chelators diminished both the alcohol-induced $[Ca^{2+}]_i$ increases and cell loss. These findings are not only in agreement with earlier studies that linked alcohol-induced $[Ca^{2+}]_i$ elevations with cell death (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005, Hirata et al. 2006), but also extend these studies by identifying the Ca^{2+} surge as originating from intracellular stores and by establishing that alcohol initiates the neuronal death that occurs much later within moments of exposure. Although the mechanisms by which the alcohol-induced rise in $[Ca^{2+}]_i$ triggers cell death are still under investigation, our initial examination assessed two cytosolic and mitochondrial Ca^{2+} activated pathways. Inhibiting either calcineurin or CaMKii, which are Ca^{2+} -activated cytosolic components of separate apoptotic pathways, shielded CGN cultures from alcohol toxicity. Blocking the MCU and the MPTP also ameliorated alcohol-induced neuronal loss and implicate the mitochondria. Thus, it appears that Ca^{2+} initiates multiple parallel apoptotic signaling cascades that synergistically interact to precipitate cell death and likely explains why inhibition of one of many these pathways can protect neurons from alcohol toxicity. These results imply that a therapeutic intervention which can counteract alcohol's disruption of Ca^{2+} homeostasis may alleviate the severe neuronal loss that accompanies alcohol exposure in the developing brain.

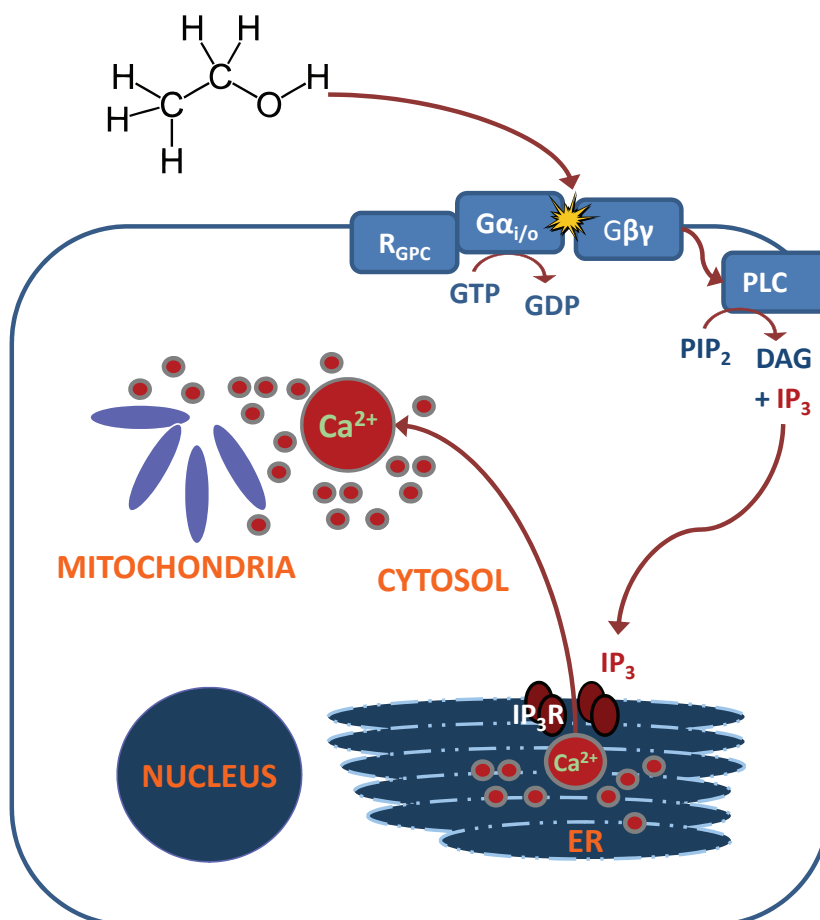


Figure II-1. A visual representation of our hypotheses. We hypothesized that ethanol induces cell death in CGN cultures by stimulating a rapid $[Ca^{2+}]_i$ increase via PLC-dependent formation of IP_3 . IP_3 activates the IP_3R , triggering Ca^{2+} release from internal stores (ER). We propose that this intracellular Ca^{2+} increase is a key trigger for alcohol-induced neuronal death.

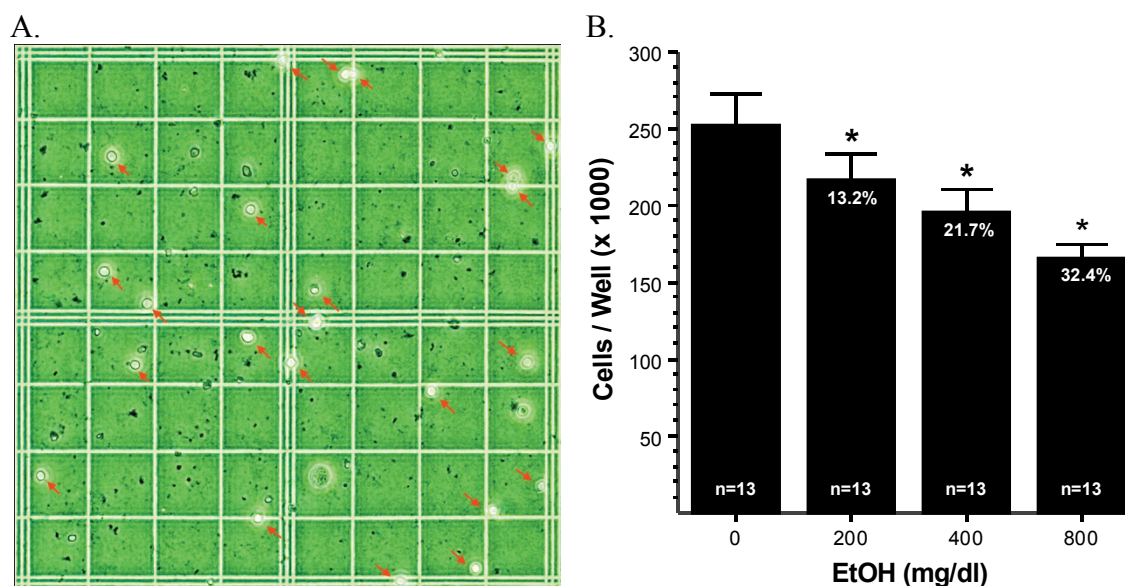
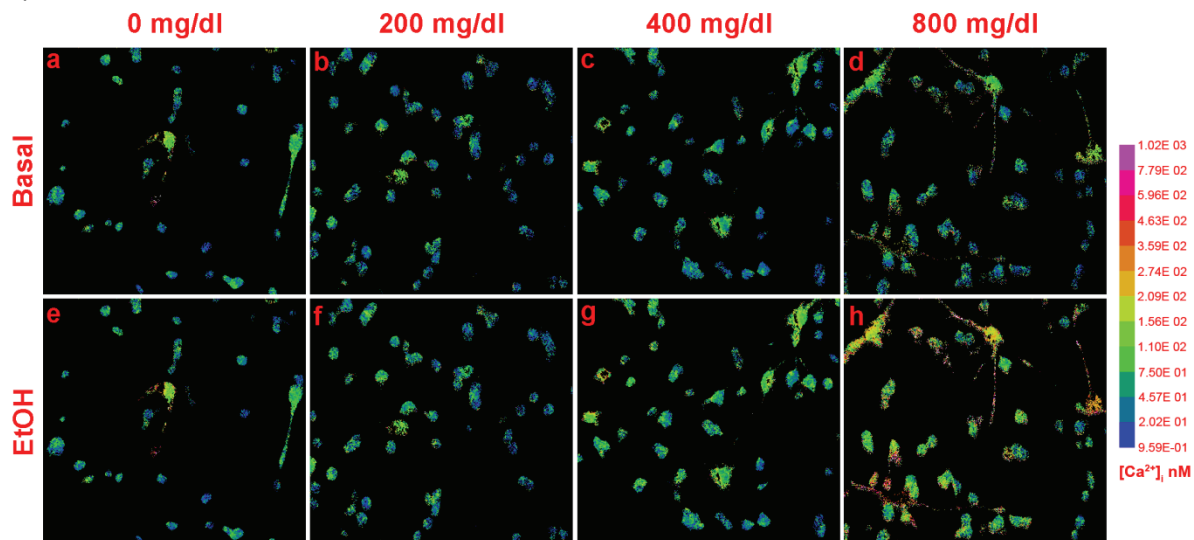


Figure II-2. Alcohol reduces cell number in CGN primary cultures. CGN cultures were exposed to either 0, 200, 400, or 800 mg/dl ethanol. After a 24-hour alcohol exposure, viable cells (trypan blue exclusion) were counted on a hemacytometer. Identified in Panel A are viable CGNs. Viable cells exclude trypan blue and show a characteristic phase contrast halo used in all CGN viability studies through orange arrows. Panel B demonstrates the alcohol dose-dependent reduction of viable CGN neurons. Values represent the mean number of cells/well \pm SEM (standard error of the mean). Percent cell loss is shown at the top of each bar. Significant differences ($p < 0.05$) from no-alcohol (0 mg/ml) cultures are indicated by *. A 24-hour alcohol exposure at 200, 400, or 800 mg/dl significantly reduced cell numbers by 13.2%, 21.7%, or 32.4% respectively.

Figure II-3. Alcohol induces a rapid rise in intracellular Ca^{2+} . Panel A shows representative pseudocolor 340/380 ratio images of Fura-2 fluorescence in CGN cultures derived from a single mouse litter. The colored scale bar (right) represents $[\text{Ca}^{2+}]_i$ with blue and red depicting low and high $[\text{Ca}^{2+}]_i$, respectively. Panels a through d (above) show fields from each alcohol-treatment group before alcohol exposure, whereas panels e through f (below) are the identical fields viewed one minute after initiating alcohol exposure at the dose indicated (0, 200, 400, or 800 mg/dl). With increasing doses of alcohol, more neurons show substantial increases in $[\text{Ca}^{2+}]_i$. For comparison, view the same neuron prior to alcohol exposure in the panel above. In Figure II-3B, each Ca^{2+} trace combines data from four mouse litters, representing all cells that received the specified ethanol dose. The displayed values in the traces show the mean $[\text{Ca}^{2+}]_i \pm \text{SEM}$ in each cell population every 5 seconds for 5 minutes. Addition of 800 mg/dl ethanol induces a rapid $[\text{Ca}^{2+}]_i$ surge, whereas 400 mg/dl initiated a more gradual rise, both attaining a mean of 65 nM above basal $[\text{Ca}^{2+}]_i$ levels (~ 65 nM) within 180 seconds. Ethanol exposure at 200 mg/dl increased $[\text{Ca}^{2+}]_i$ to 90 nM. Greater alcohol concentrations yielded both a more rapid rise and a higher increase in $[\text{Ca}^{2+}]_i$.

A.



B.

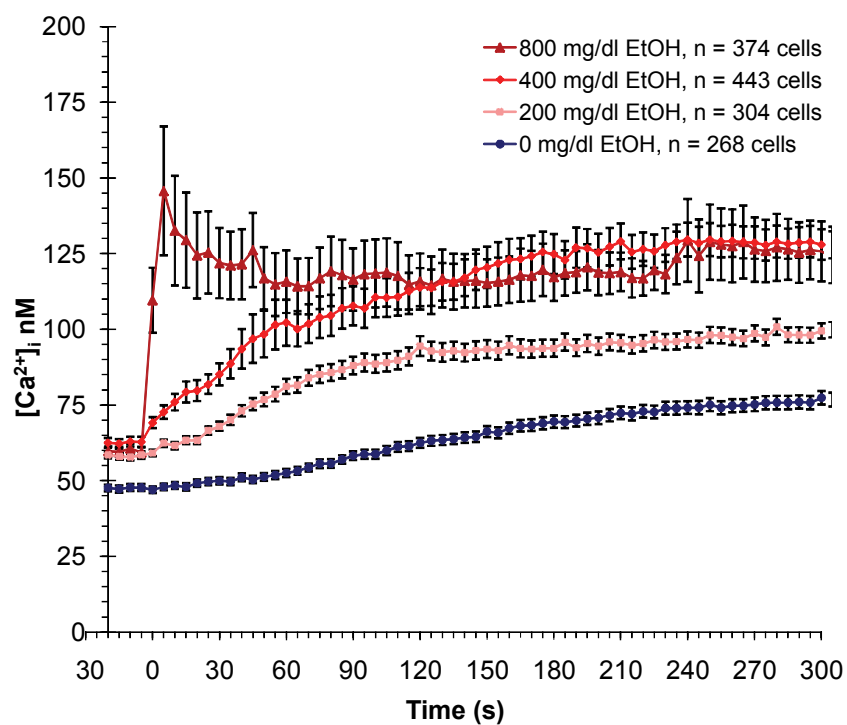
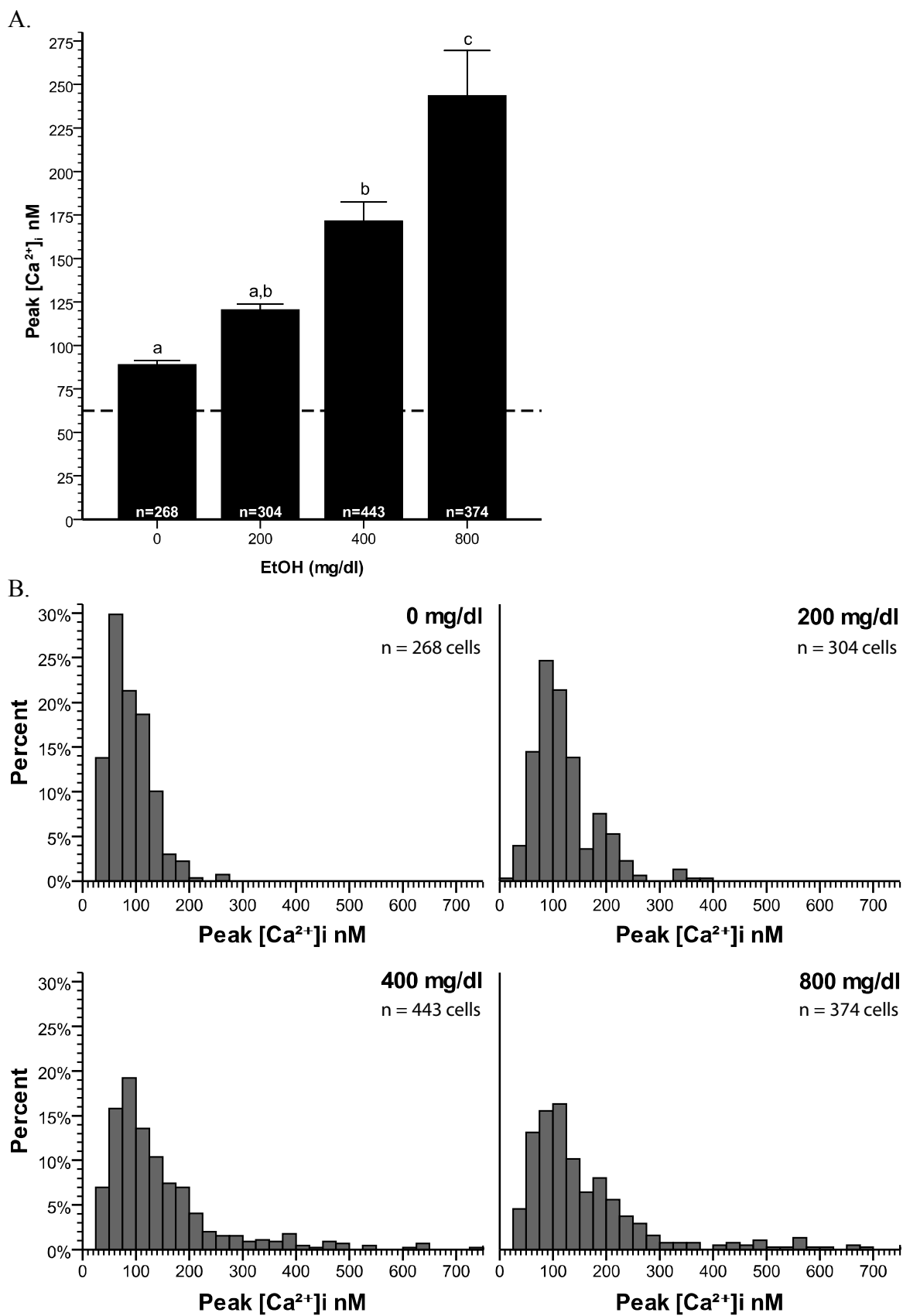


Figure II-4. Alcohol's peak elevation of intracellular Ca^{2+} is dose-dependent. Figure II-4A shows summary data (mean \pm SEM) pooled from the four mouse litters in Figure II-3. For every cell, the peak $[\text{Ca}^{2+}]_i$ level corresponds to the highest $[\text{Ca}^{2+}]_i$ value attained over the 5 minutes following alcohol addition. Cells were stimulated with increasing doses of ethanol (0, 200, 400, 800 mg/dl). For each ethanol concentration, the bars represent the mean \pm SEM of the peak $[\text{Ca}^{2+}]_i$ levels attained for all cells in that ethanol dose group. The dashed line indicates the baseline $[\text{Ca}^{2+}]_i$ level, which was calculated as the mean unstimulated $[\text{Ca}^{2+}]_i$ for all the cells in this experiment. Unstimulated basal $[\text{Ca}^{2+}]_i$ levels for each cell was calculated by averaging the $[\text{Ca}^{2+}]_i$ measured in the 15 seconds preceding alcohol exposure. Statistical differences were determined by one-way ANOVA and Scheffé post-hoc paired t-tests. Means that share a letter (a, b, or c) are not significantly different. Means without a shared letter differ significantly ($p < 0.05$). In Figure II-4B, the peak neuronal $[\text{Ca}^{2+}]_i$ levels for each alcohol treatment condition were grouped into 20 nM bins and plotted in the histograms. As can be seen, alcohol increased the range of the peak Ca^{2+} response.



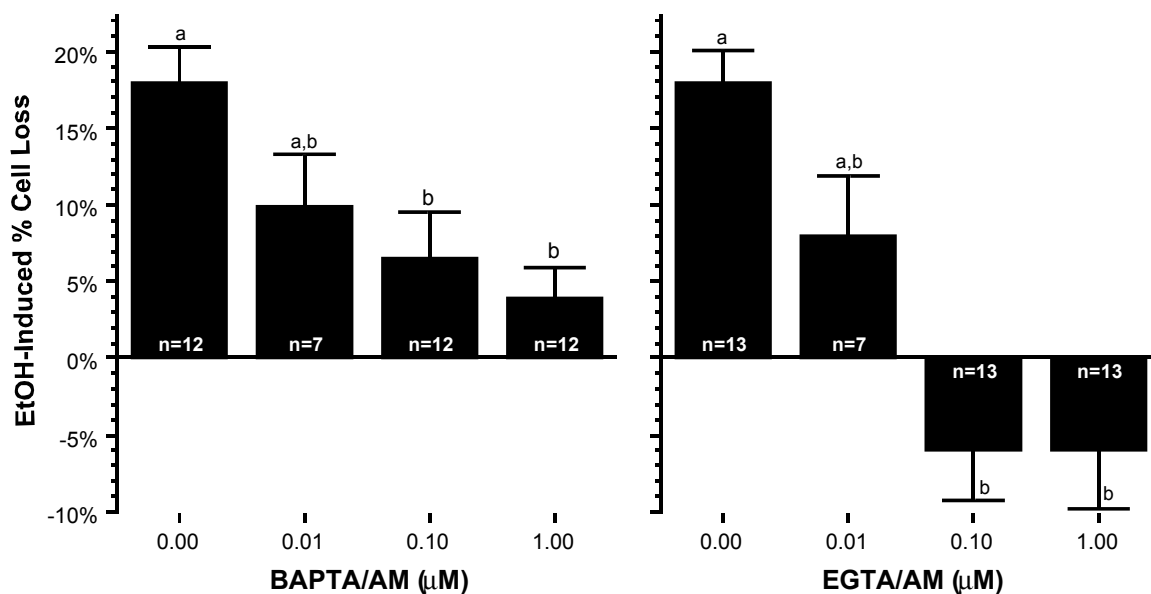
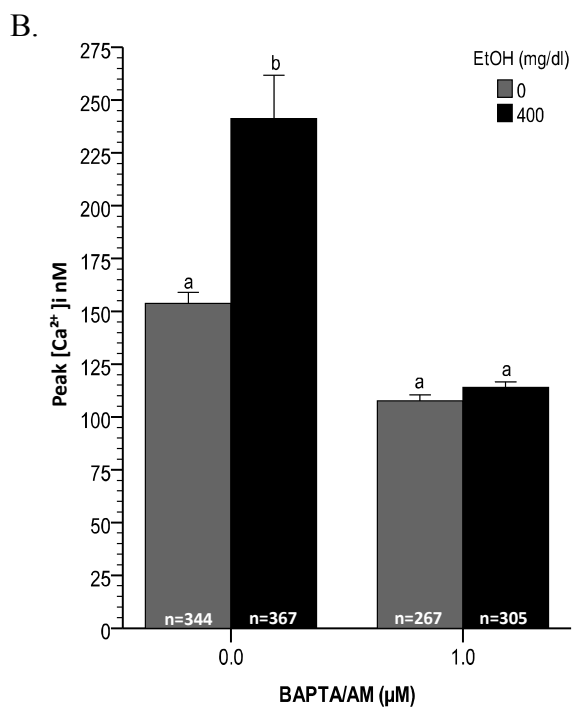
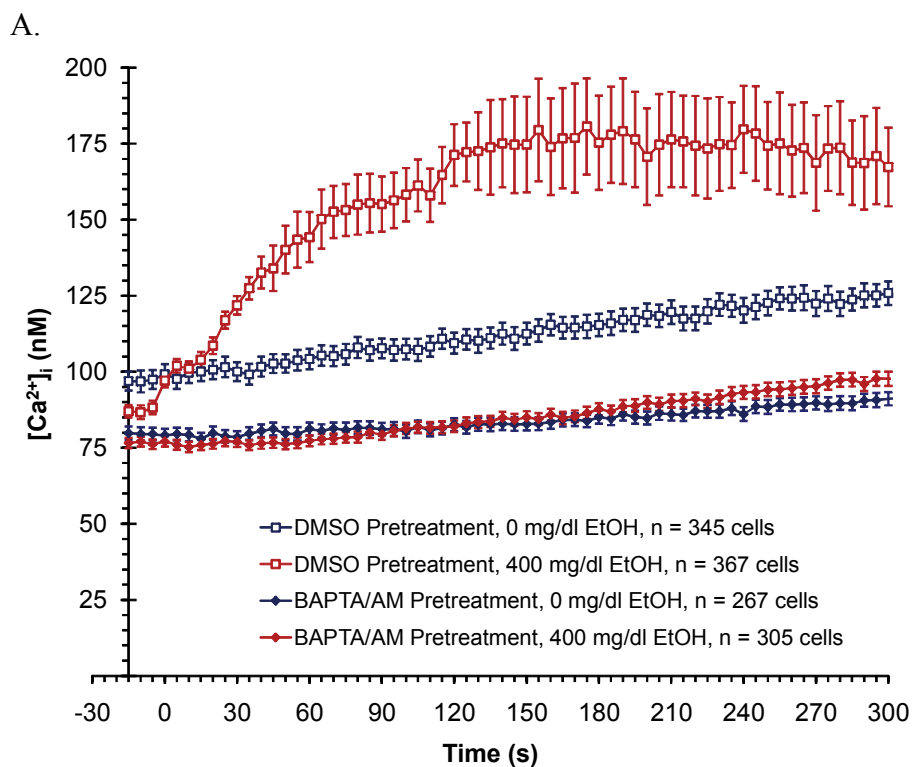


Figure II-5. Membrane-permeable Ca^{2+} chelators reduce alcohol-induced neuronal death in CGN cultures. Ethanol-induced percent cell loss was calculated by comparing ethanol-exposed groups to ethanol-free cultures similarly pretreated with BAPTA/AM or EGTA/AM. Means that share a letter (a or b) are not significantly different. Means without a shared letter differ significantly as determined by one-way ANOVA and Scheffé post-hoc tests ($p < 0.05$). Alcohol exposure in the absence of a Ca^{2+} chelator caused an 18% neuronal loss in CGN cultures. Pretreatment with membrane-permeable Ca^{2+} chelators (BAPTA/AM or EGTA/AM) reduced alcohol-induced cell death to below 5% at the higher doses of either chelator. The neuroprotective effect of both agents was dose-dependent.

Figure II-6. BAPTA/AM abolishes the alcohol-induced rise in $[Ca^{2+}]_i$. CGN cultures were pretreated with the membrane-permeable Ca^{2+} chelator BAPTA/AM (1.0 μ M) for 30 minutes before exposure to ethanol (400 mg/dl) or PBS. In Figure II-6A, displayed values signify mean $[Ca^{2+}]_i$ levels in each cell population tested (5 litters). Measurements from BAPTA/AM-pretreated cultures are shown with lines with filled-in shapes, whereas lines representing cultures with DMSO vehicle pretreatment contain hollow shapes. Red lines represent CGN cultures that received EtOH additions, whereas blue lines represent PBS additions. Baseline $[Ca^{2+}]_i$ values were determined for 15 seconds prior to the exposure time (time = 0). In the same cell cultures, the bars in Figure II-6B show the peak $[Ca^{2+}]_i$ attained over the 5 minutes following alcohol addition (0 or 400 mg/dl). Means without a shared letter differ significantly ($p < 0.05$).



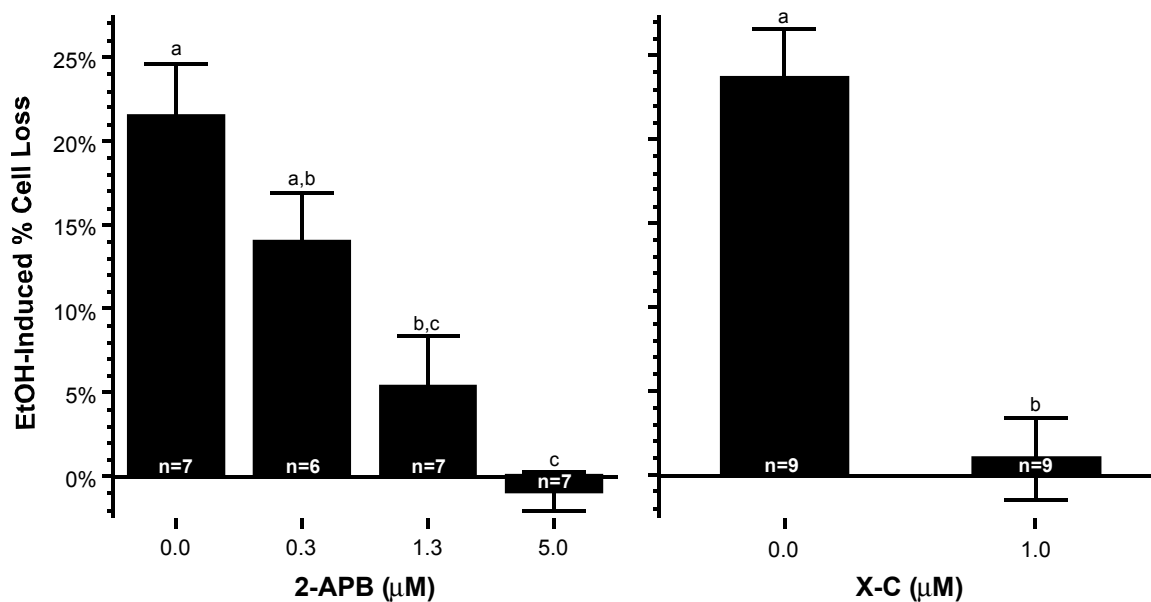


Figure II-7. IP₃R inhibitors, which block Ca²⁺ release from internal stores, eliminate alcohol-induced cell death in CGN cultures. Primary CGN cultures were pretreated for 30 minutes with membrane-permeable IP₃R inhibitors (either 2-APB or X-C) prior to initiating a 24-hour alcohol exposure (400 mg/dl). Ethanol-induced percent cell loss was calculated by comparing ethanol-exposed groups with ethanol-free cultures receiving the identical pretreatment dose of 2-APB or X-C. Scripts are defined in Figure II-5. Blocking intracellular Ca²⁺ release from internal stores with IP₃R inhibitors has a strong neuroprotective effect, eliminating alcohol-induced neuronal loss.

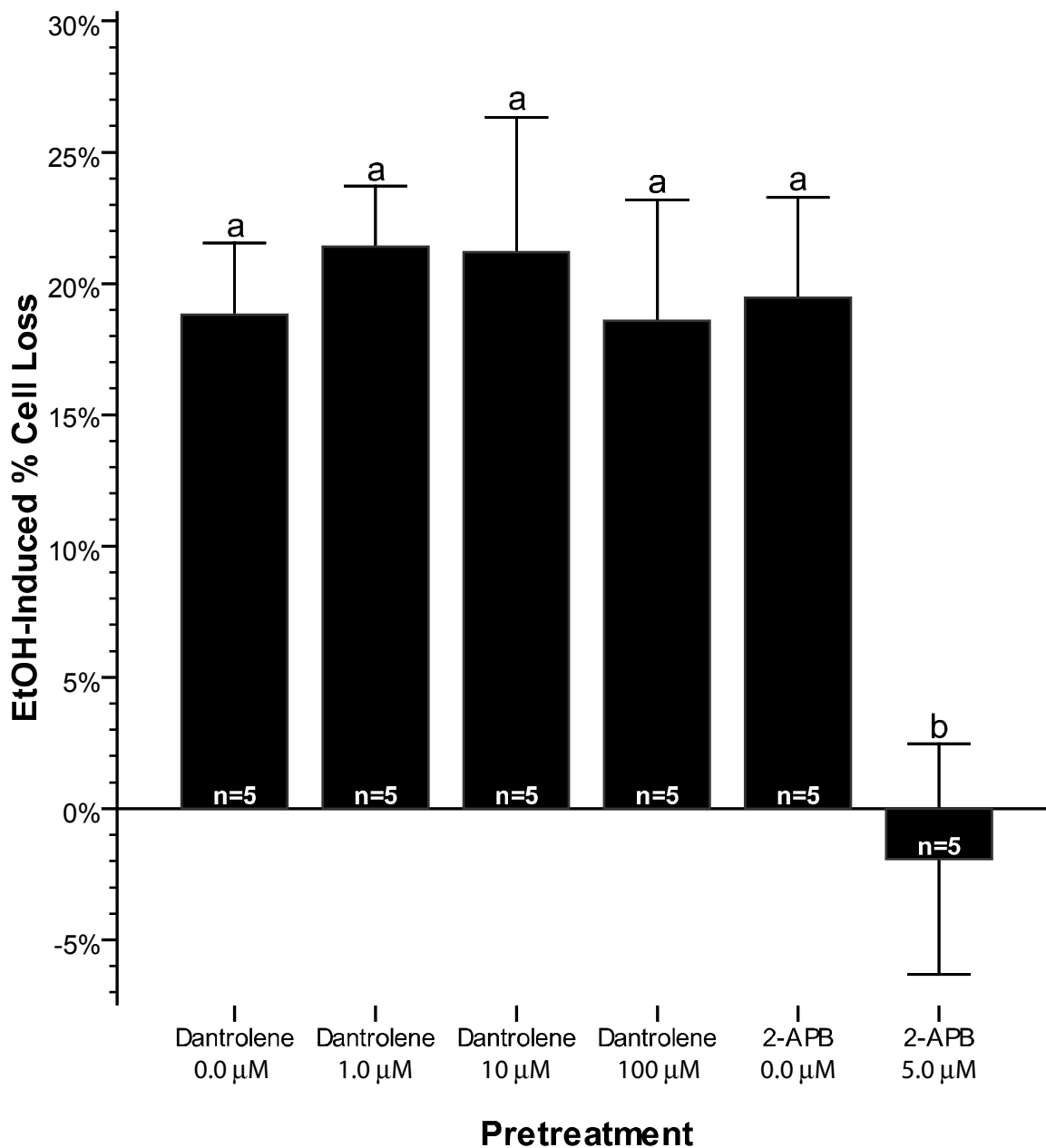
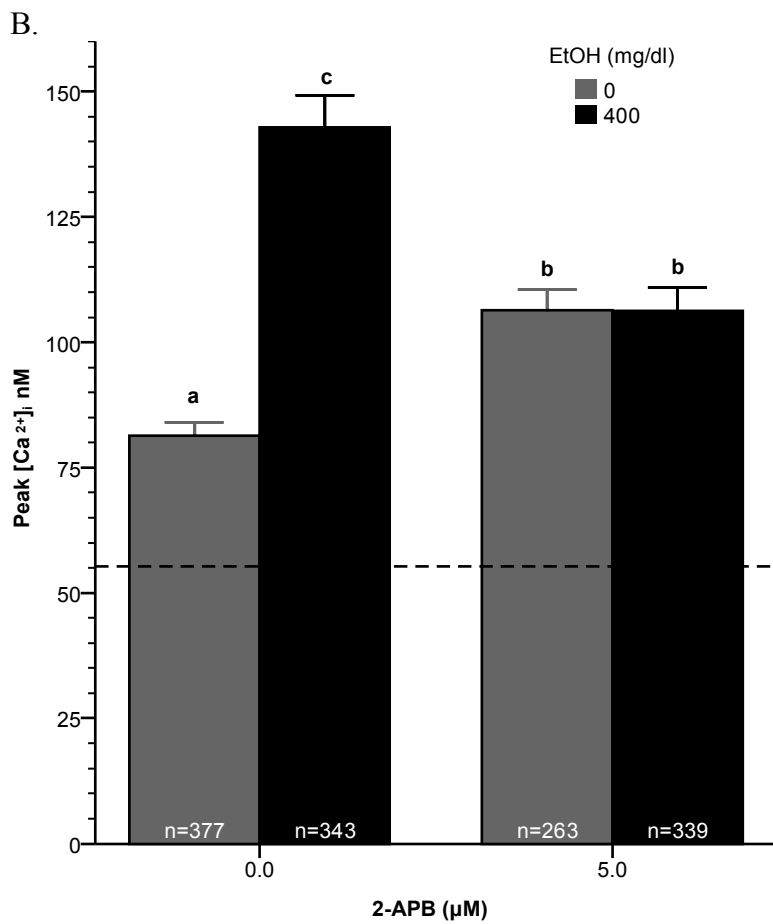
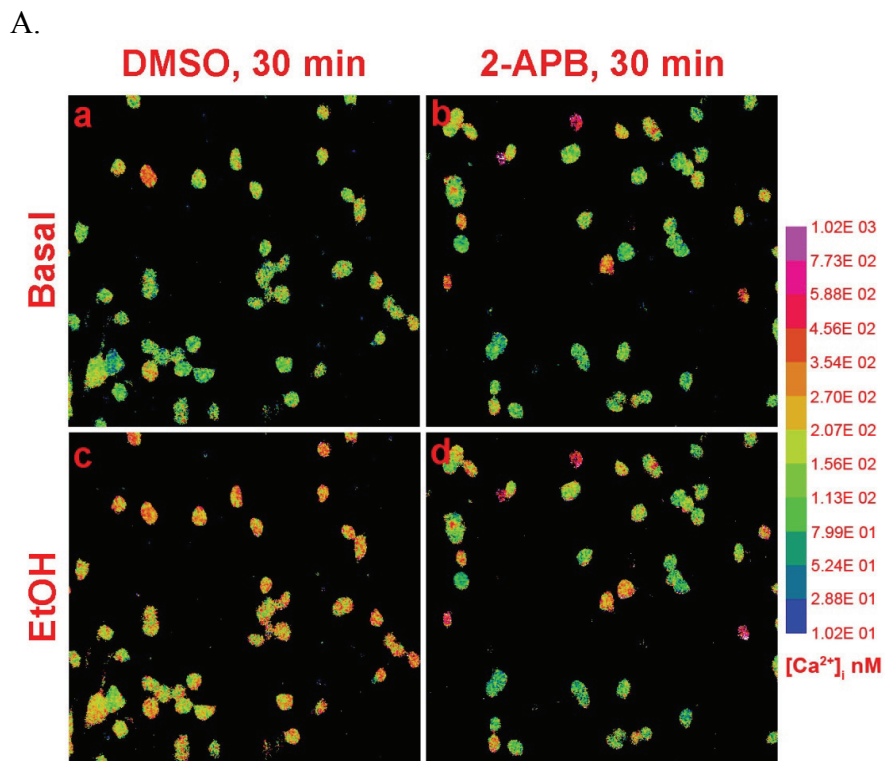


Figure II-8. Blocking IP₃R_s but not RyR_s protects CGN cultures from alcohol-induced cell death. Thirty minutes before initiating a 24-hour alcohol exposure (400 mg/dl), CGN cultures were pretreated with either a membrane-permeable RyR inhibitor (dantrolene) or an IP₃R inhibitor (2-APB). Scripts (a or b) are defined in Figure II-5. No significant protection from alcohol exposure is observed by blocking RyR_s with dantrolene. This signifies that IP₃R_s rather than RyR_s are involved in alcohol-induced neuronal death.

Figure II-9. 2-APB blocks the rapid ethanol-induced rise in $[Ca^{2+}]_i$ in CGN cultures. Illustrated in Figure II-9A are representative photomicrographs of the data collected in this experiment. Panels a - b depict fields from each treatment groups before alcohol exposure (400 mg/dl), whereas panels c - d are the identical fields viewed approximately one minute after initiating alcohol exposure. Panels a and c (left) portray DMSO pretreatment, whereas panels b and d (right) show 2-APB pretreatment (5 μ M). The colored scale bar (far right) depicts $[Ca^{2+}]_i$ with blue and red corresponding to low and high $[Ca^{2+}]_i$, respectively. To visualize $[Ca^{2+}]_i$ changes, compare the color of the neurons in the panels above (prior to alcohol exposure) to identical neurons in the panels below. Less neurons reveal alcohol-induced increases in $[Ca^{2+}]_i$ with 2-APB pretreatment. Illustrated in Figure II-9B are $[Ca^{2+}]_i$ traces from the same CGN cultures that were pretreated with IP₃R inhibitor, 2-APB (5 μ M), or HBSS (control) for 30 minutes prior to initiating ethanol exposure.



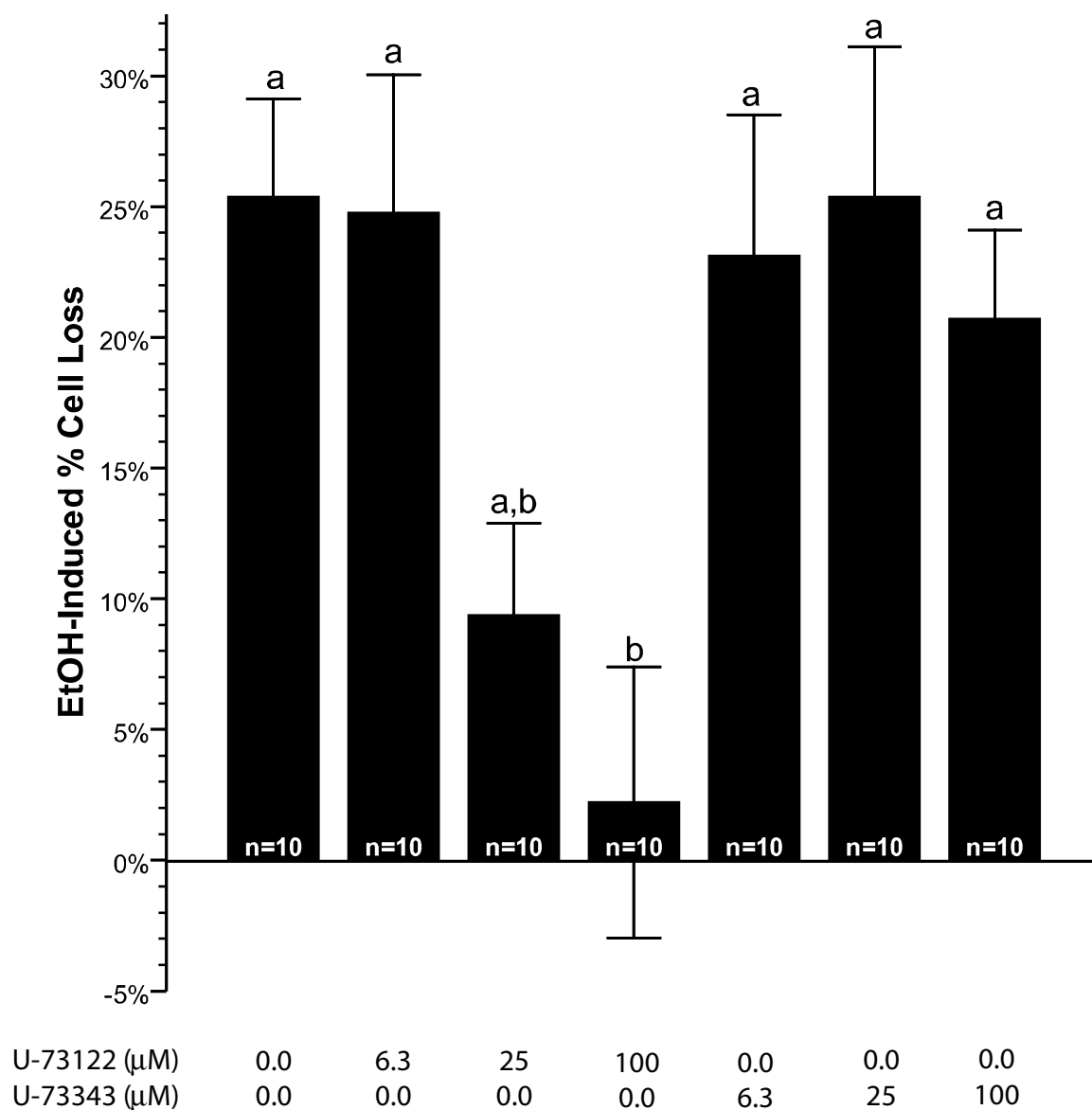
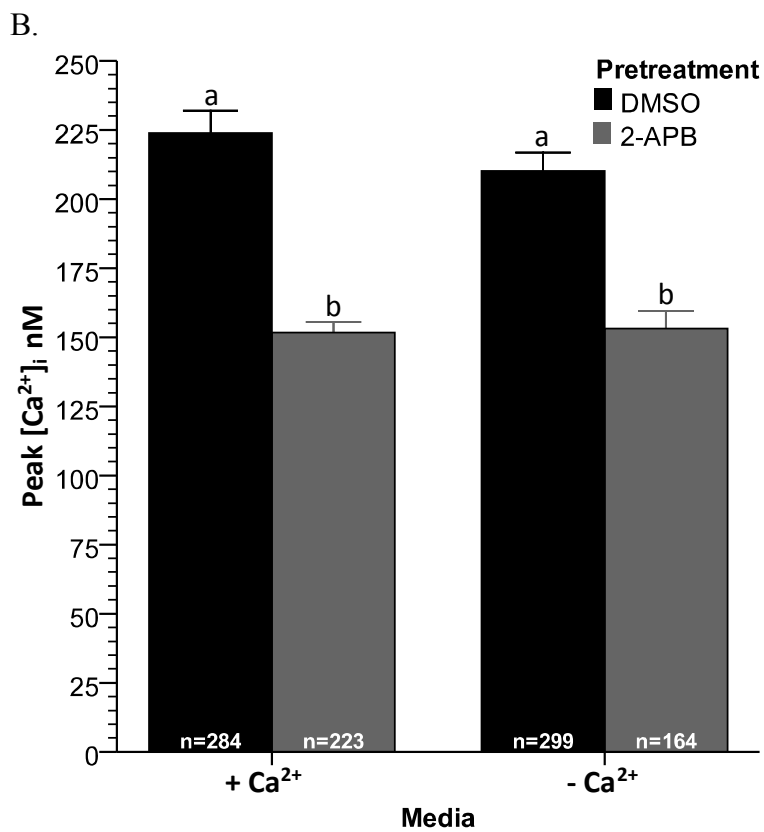
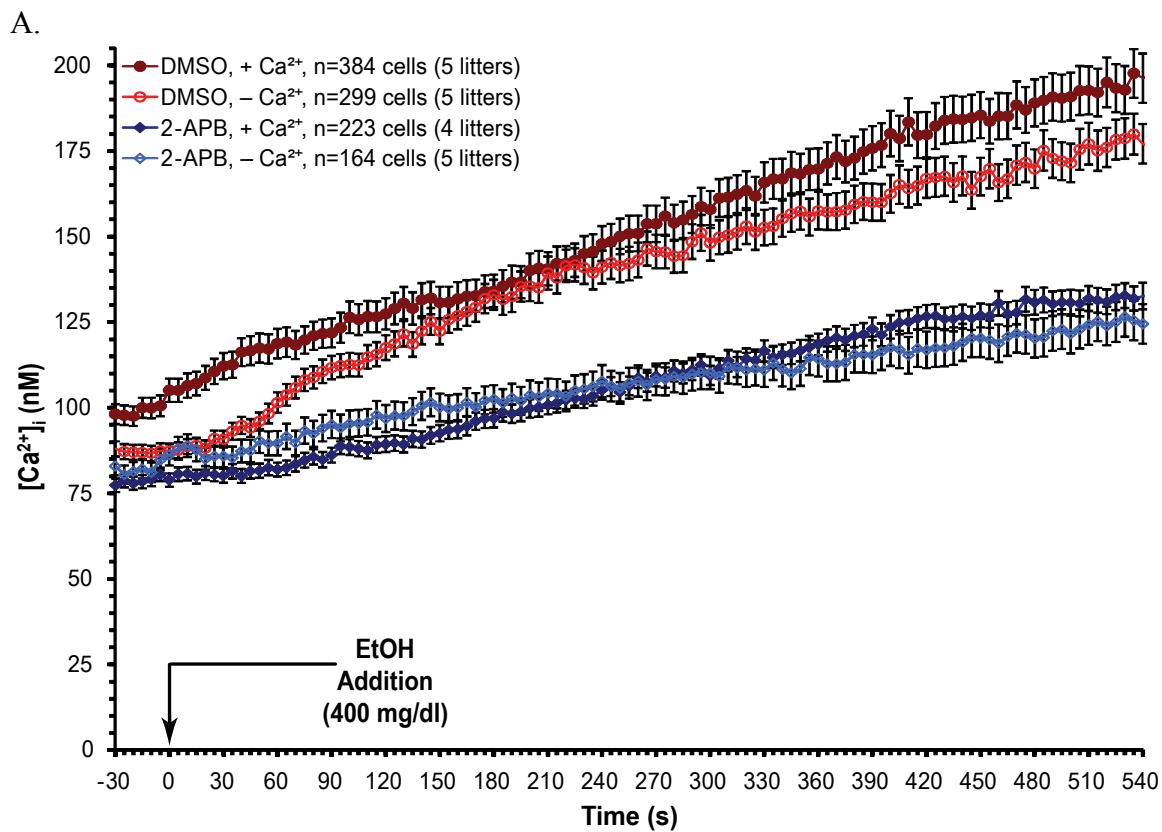


Figure II-10. Inhibition of PLC protects CGN cultures from alcohol-induced neuronal death. Prior to 24-hour ethanol exposure (400 mg/dl), CGN cultures were treated with either a PLC inhibitor (U-73122) or its respective inactive analog (U-73343). Means without a shared letter (a or b) differ significantly as indicated by Tukey post-hoc tests ($p < 0.05$). U-73122, but not U-73343, brought alcohol-induced neuronal death to lower than 5%, indicating that inhibiting PLC strongly protects CGN cultures from alcohol toxicity.

Figure II-11. Removal of Ca^{2+} from media does not abolish the alcohol-induced rise in $[\text{Ca}^{2+}]_i$ in CGN cultures. Primary CGN cultures were pretreated with the cell-permeable IP_3R inhibitor, 2-APB (5 μM), or DMSO (vehicle control) for 30 minutes prior to initiating alcohol exposure. Immediately prior to imaging, CGN-containing coverslips were transferred to HBSS media containing either Ca^{2+} (+ Ca^{2+}) or not (– Ca^{2+}) and either 2-APB or DMSO as indicated in the legend of Figure II-11A. Baseline $[\text{Ca}^{2+}]_i$ values were determined for 15 seconds prior to the addition of ethanol (400 mg/dl) at time = 0 seconds. The bars in Figure II-11B show the peak $[\text{Ca}^{2+}]_i$ attained during 9 minutes after alcohol addition. Means without a shared letter differ significantly ($p < 0.05$). Regardless of whether Ca^{2+} was present in the media, EtOH promptly increased $[\text{Ca}^{2+}]_i$ in the absence of 2-APB. 2-APB eliminated ethanol-induced rise of $[\text{Ca}^{2+}]_i$. This suggests the observed elevation in $[\text{Ca}^{2+}]_i$ from ethanol exposure originates from an intracellular source instead of an extracellular one.



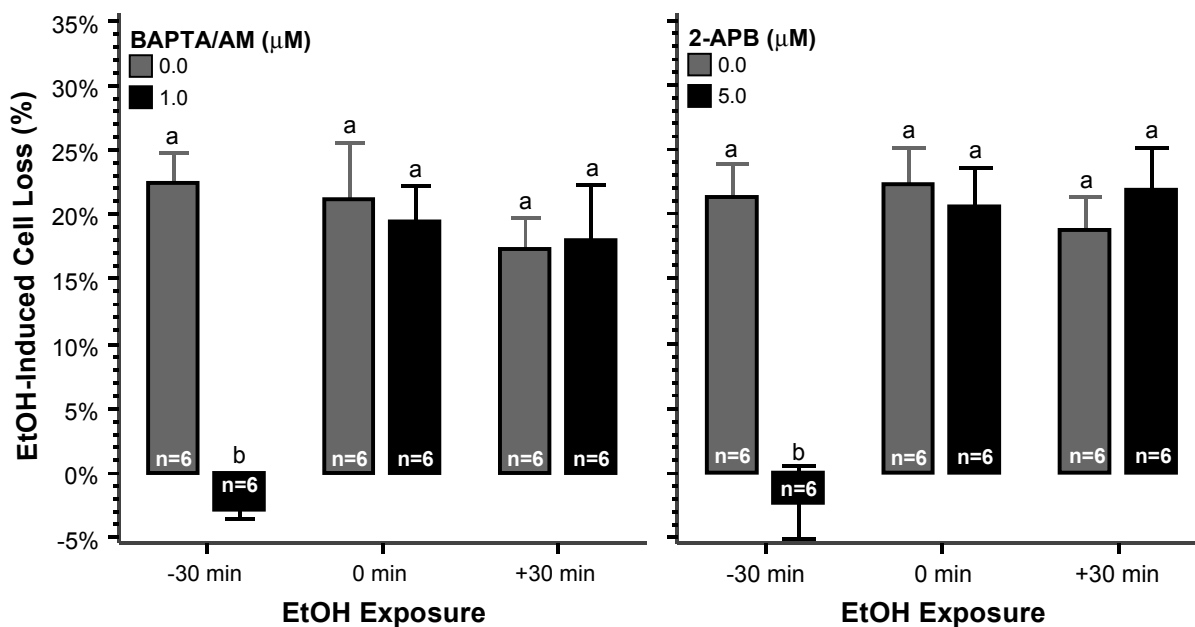


Figure II-12. Ca^{2+} chelation or IP_3R inhibition must precede ethanol exposure to be neuroprotective. The figure shows ethanol-induced percent cell loss when CGNs are treated with Ca^{2+} chelator, BAPTA/AM, or IP_3R blocker, 2-APB, either 30 minutes before, simultaneously with, or 30 minutes after alcohol exposure (400 mg/dl) has begun. Neither BAPTA/AM nor 2-APB was neuroprotective when added simultaneously with alcohol or 30 minutes after alcohol exposure has begun. Only a 30 minute pretreatment before initiating alcohol exposure prevented the alcohol-induced neuronal death. The letters (a, b) are explained in Figure II-5.

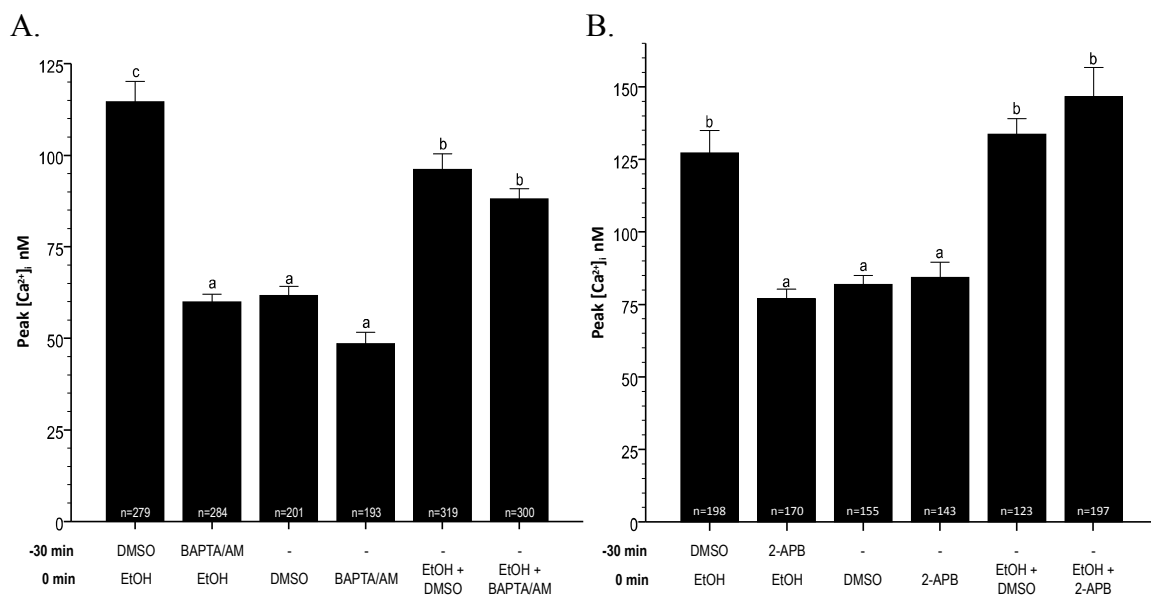


Figure II-13. BAPTA/AM and 2-APB must precede alcohol exposure to prevent the rapid ethanol-induced $[Ca^{2+}]_i$ in CGN cultures. Bars represent the mean peak $[Ca^{2+}]_i$ attained over five minutes for each cell population as measured by Fura-2 fluorescence. The x axes show the additions made to the culture media 30 minutes before (-30 min) and at the start (0 min) of calcium measurements. Each graph shows the results pooled from 5 litters for panel A and 3 litters for panel B. Means without a common letter (a, b, c) differ significantly ($p < 0.05$). Unlike when these agents are added prior to ethanol exposure (the second bar of each graph), simultaneous addition failed to prevent the alcohol-induced increase in $[Ca^{2+}]_i$ (the last bar of each graph).

Figure II-14. Inhibition of two different downstream calcium effectors protects CGN cultures from alcohol-induced neuronal death. Thirty minutes before initiating 24-hour ethanol exposure, CGN cultures were treated either with a calcineurin inhibitor (FK-506) as shown in panel A, and either a CaMKii inhibitor (KN-93) or its inactive analog (KN-92) as shown in panel B. Means without a shared letter (a, b, or c) differ significantly as determined by one-way ANOVA and Scheffé post-hoc tests ($p < 0.05$). In the case of each of these two downstream calcium effectors, their inhibition protected against alcohol-induced cell loss. This neuroprotection is similar to that observed by PLC inhibition (Figure II-10), IP₃R blockade (Figure II-7), and by Ca²⁺ chelation (Figure II-5).

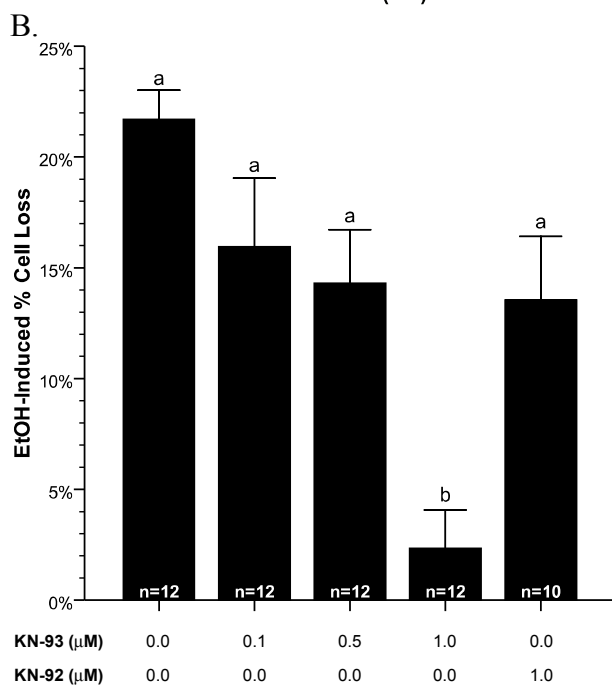
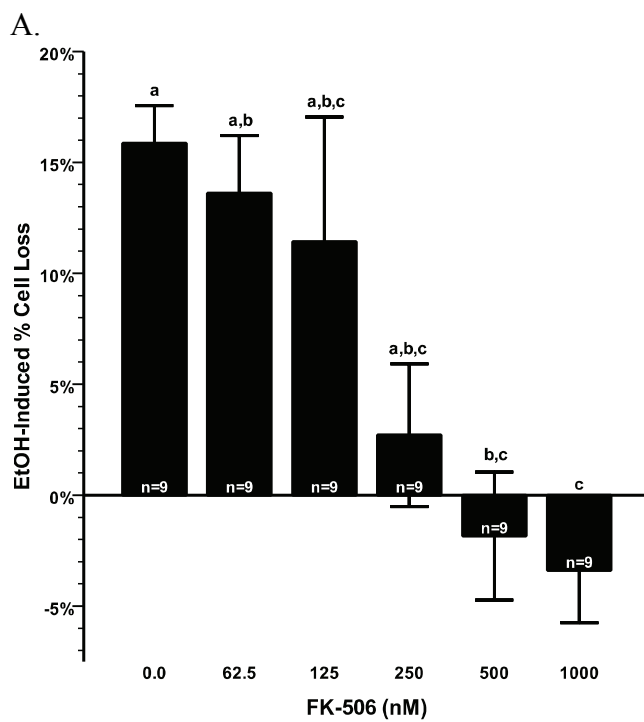
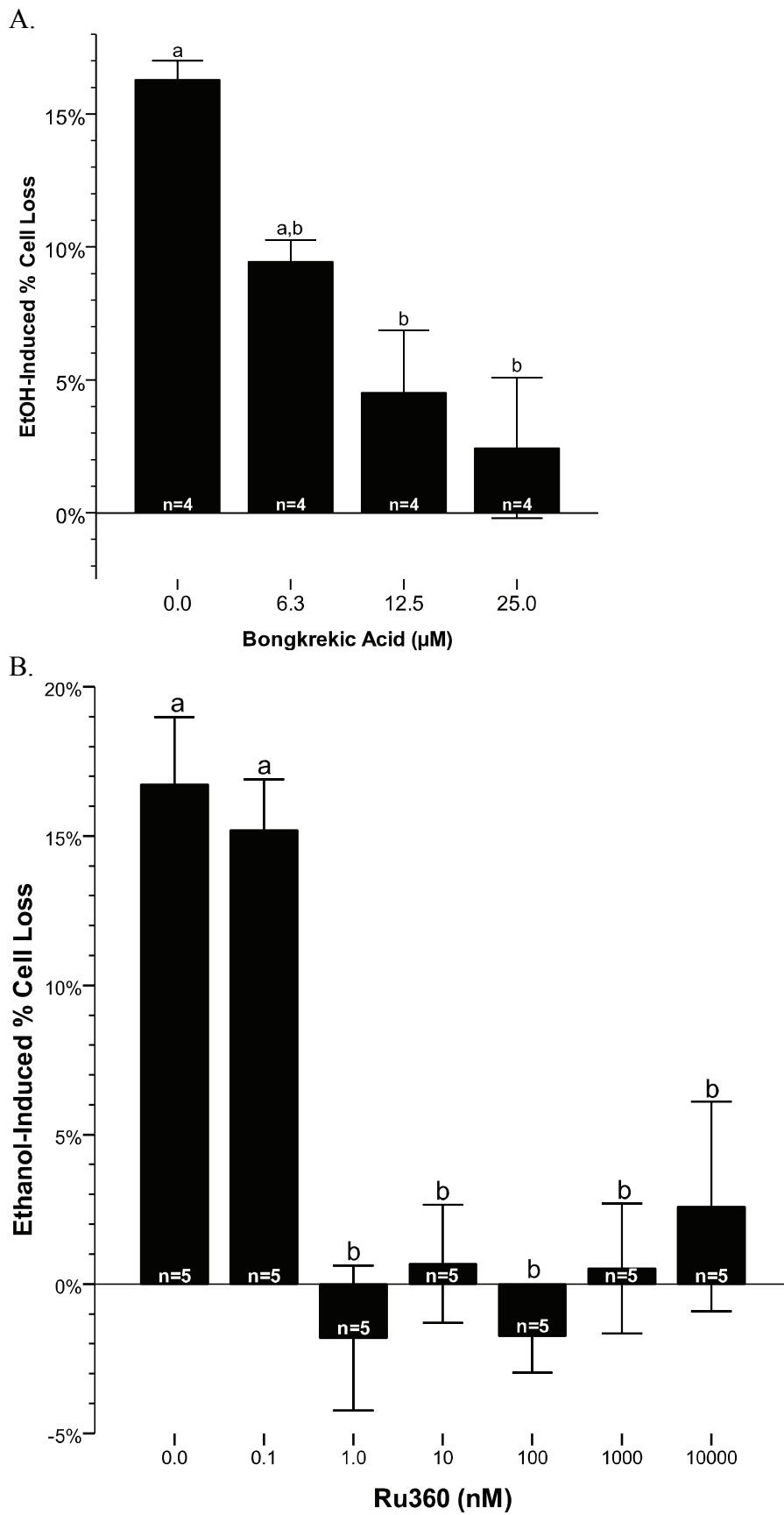


Figure II-15. Mitochondrial calcium uniporter or MPTP inhibition protects CGN cultures from alcohol-induced neuronal death. Thirty minutes before initiating 24-hour ethanol exposure, CGN cultures were treated either with MPTP inhibitor (bongkreikic acid) as shown in Panel A or with a mitochondrial Ca^{2+} uniporter inhibitor (Ru360) as shown in Panel B. Means without a shared letter (a, b, or c) within each panel differed significantly as determined by one-way ANOVA and Scheffé post-hoc tests ($p < 0.05$). In the each case, inhibition protected against alcohol-induced cell loss.



CHAPTER III

THE NITRIC OXIDE SIGNALING PATHWAY BLOCKS ALCOHOL-INDUCED INTRACELLULAR CALCIUM RELEASE

Background and Significance

FASD remains the single leading preventable cause of mental retardation in the United States (Riley & McGee 2005). Prenatal alcohol exposure generates widespread neuronal loss, that varies in severity among brain regions (Konovalov et al. 1997). Since these brain abnormalities culminate in serious behavioral and neuropsychological deficits that persist into adulthood, (Hannigan & Armant 2000), achieving better understanding of the factors underlying neuronal alcohol vulnerability is of prime importance.

In our neuronal cell-culture model of FASD, NMDA lessened the alcohol vulnerability of CGN cultures by activating nNOS, and increasing intracellular NO levels (Pantazis et al. 1995). NO stimulates sGC, an enzyme that synthesizes cGMP (Moncada & Higgs 1995), which in turn, activates PKG (Francis & Corbin 1999). Prior studies indicated that activation of either sGC or PKG diminishes alcohol-induced cell death in CGN cultures, while their inhibition blocks NO-mediated neuroprotection (Pantazis et al. 1998, Bonthius et al. 2003). Subsequent *in vivo* work also showed that alcohol exposure in neonatal wild-type mice produced far less cerebellar damage than in nNOS^{-/-} mice (Bonthius et al. 2002, Bonthius *et al.* 2006), further verifying, but now *in vivo*, the protective effect of NO against alcohol toxicity. In terms of the NO pathway providing physiological alcohol protection, nNOS expression begins increasing in the wild-type rat cerebellum starting on postnatal day 7 (Wang et al. 1998), which coincides with the development of alcohol resistance (Karaçay *et al.* 2008, Goodlett & Eilers 1997). Likewise, NOS inhibition in adult rats exacerbated ethanol-induced neurodegeneration (Zou et al. 1996).

Recently, we have focused our attention on downstream targets for PKG that would transmit the neuroprotective signal provided by activation of the NO signaling pathway. Since disruption of Ca^{2+} homeostasis commonly triggers cell death, the NO signaling pathway may aid in regulating intracellular Ca^{2+} levels. Indeed, studies in neonatal mouse astrocyte cultures (Holownia et al. 1997, Hirata et al. 2006), and in chick embryo neural crest (Garic-Stankovic et al. 2005, Debelak-Kragtorp et al. 2003) have demonstrated that alcohol elevates intracellular Ca^{2+} while inducing cell death, and that these elevations require IP_3R -dependent Ca^{2+} release from intracellular stores (Garic-Stankovic et al. 2005). This is also consistent with the observations made in CGN cultures presented in Chapter II. The IP_3Rs , as a major conduit for Ca^{2+} release from intracellular stores, are also strategically located and functionally downstream of PKG. PKG has been observed to regulate intracellular Ca^{2+} (Felbel *et al.* 1988) by phosphorylating (Haug et al. 1999) and consequently inhibiting the IP_3R (Schlossmann et al. 2000).

The principal goal of this study was to understand how NO activation of PKG downstream counters developmental alcohol toxicity. Evidence of alcohol's disruption of calcium homeostasis along with PKG's ability to regulate IP_3R -dependent Ca^{2+} release has led us to examine how pharmacological manipulation of PKG affects alcohol-induced neuronal death and intracellular calcium homeostasis. We hypothesized that cGMP activation of PKG can prevent alcohol-induced increases in $[\text{Ca}^{2+}]_i$ and this effect is linked to the neuroprotection provided by the NO signaling pathway.

Materials and Methods

With the exception of the pharmacological agents used, all methodology here was identical that outlined in Chapter II.

Cell Culture

Primary CGN cultures were established from wild-type neonatal mice (5 - 7 days old). Cerebella harvested from all the pups of each litter were combined in HEPES-buffered Earle's balanced salt solution (HEBSS), minced, and then trypsinized (Sigma T-8253, 1.25 mg/ml) for 15 minutes at 37°C. To inactivate trypsin, trypsin inhibitor (Sigma T-9003, 0.8 mg/ml final) and deoxyribonuclease-I (Sigma D-5025, 250 Kunitz units/ml final) were added before a brief centrifugation to sediment tissue. To dissociate cells, the resulting pellet was resuspended and triturated in HEBSS containing trypsin inhibitor (5.8 mg/ml) and deoxyribonuclease-I (1800 Kunitz units/ml). After centrifuging this suspension through 4% bovine serine albumin, the pellet was resuspended and triturated in N-2 supplemented DMEM to produce a cell suspension for plating. These procedures yield cell cultures comprised of 95% CGN (Dutton 1990).

For cell survival studies, cells were cultured on PDL-coated (50 mg/ml) 96-well microplates at a density of 1.5×10^6 cells/ml (300 μ l/well). For each litter, duplicate or triplicate cultures were plated at each dose of pharmacological agent to be added later. CGN cultures in ethanol-exposed and ethanol-free groups were maintained on separate plates. For Ca^{2+} measurement studies, cell suspensions were deposited into the individual wells of 6-well microplates which contained PDL-coated (100 mg/ml) glass coverslips (25 mm in diameter). Cells were plated at a density of 1.0×10^6 cells/ml (2.0 ml/well).

For all experiments, CGN cultures were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2 / 95% air prior to use.

Pharmacological Treatments and Ethanol Exposure

Pharmacological agent and ethanol exposure was initiated 24 hours after plating. Thirty minutes prior to, simultaneously, or thirty minutes after initiating ethanol exposure (400 or 800 mg/dl, final concentration), cultures were treated with one or more of the following agents: 1. PBS (vehicle control); 2. DETA-NONOate, a NO donor that

activates sGC; 3. 8-Br-cGMP, a nonhydrolyzable cGMP analogue that activates PKG; 4. Rp-8-pCPT-cGMPS, a PKG inhibitor; BDNF, a neurotrophin that provides alcohol neuroprotection via a non-NO signaling pathway, 6. 2-APB, an IP₃R inhibitor that blocks intracellular Ca²⁺ release. In the experiments requiring Rp-8-pCPT-cGMPS addition, this agent was prior to 8-Br-cGMP or 2-APB. Since 2-APB requires DMSO as a solvent, non-drug treatment control cultures in toxicity studies received equivalent DMSO exposure. For pretreatment paradigms in imaging studies, unlike toxicity studies that required direct addition, pharmacological agent exposure entailed exchanging the culture media for media containing final concentration of the agent.

To prevent ethanol loss by evaporation in alcohol toxicity experiments, the ethanol-exposed tray was placed in a sealed chamber containing an ethanol bath of equal concentration to the culture media and 5% CO₂. Ethanol-free cultures were placed in containers with a water bath and 5% CO₂. In treatment paradigms requiring agent administration thirty minutes after ethanol exposure, culture trays were briefly removed from their containers to receive pharmacological agents or a vehicle control before being resealed. Chambers were incubated at 37°C for 24 hours.

Cell Viability – Trypan Blue Exclusion

CGN cultures were suspended by trituration in 0.2% trypan blue and viewed under a phase-contrast microscope (Nikon Diaphot). Viable cells were identified by two criteria: 1) dye exclusion; 2) a phase contrast halo, present on the margin of viable cells under phase-contrast microscopy. For each well, cell numbers were determined by counting viable cells on a hemacytometer (four counts per well) using a 20X objective. Within each litter, the cell numbers for each treatment condition were averaged (Pantazis et al. 1995).

Ca²⁺ Measurements – Fura-2 Fluorescence

CGN cultures on coverslips were loaded with the Ca²⁺-specific dye, Fura-2/AM (1.0 μM) for 30 minutes at 37°C. After washing with HBSS, hydrolysis of Fura-2/AM to Fura-2 occurred in the presence of the indicated agents for 30 minutes at 37°C. With fresh identical media, coverslips containing CGN cultures were transferred to a temperature-controlled chamber (37°C) mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot). Cultures then received either the addition of 95% ethanol diluted in PBS (20 μl) to the HBSS to obtain the final ethanol concentrations or a drug treatment as described. For simultaneous treatments, ethanol and pharmacological agents were prepared separately but mixed together prior to adding to the culture medium.

Using a microscopic digital imaging system (Photon Technology International), [Ca²⁺]_i levels (nM) as determined by real-time shifts in Fura-2 emission ratio fluorescence (Excitation: 340 / 380 nm) were recorded for 15 seconds before (basal) and during drug or ethanol exposure at 5 second intervals for the indicated durations (5 or 9 minutes). To compute average whole cell [Ca²⁺]_i from ratio image pairs, a mask was drawn around each cell from images captured at 340 nm excitation as previously described (Sharma et al, 1995). For each coverslip, all cells within the microscope view (an average of approximately 40 cells, ranging from 15 to 50 cells) were analyzed. In each experiment, a minimum of three litters were used.

Statistical Analyses

For cell viability experiments, each mouse litter constituted one replicate of the experiment because every mouse litter provided a single pool of cells. For each litter, the cultures in all treatment groups were established in duplicate or triplicate. The estimated numbers of viable cells in each well that received identical treatments were averaged within each litter. Since each mouse litter constitutes a single replicate, cell number data

was analyzed using a two-way or three-way repeated measures ANOVA as indicated with one factor being the presence of ethanol and others consisting of each pharmacological agent used (Bonthius et al. 2003). Follow-up post-hoc comparisons consisted of Bonferroni-corrected pairwise comparisons or paired test t-tests as directed by the repeated-measures ANOVA.

However, the changing alcohol vulnerability of CGN cultures with pharmacological additions is more easily illustrated by expressing the data as ethanol-induced cell loss instead of graphing mean well cell counts per condition (Pantazis et al. 1995). Therefore ethanol-induced cell loss was calculated as the percent ratio of the difference between the viable cells counted in each non-ethanol treatment and their respective ethanol-treated condition, over the viable cells counted in non-ethanol treatment conditions in each litter. Like the repeated-measures design, this normalization also balances the innate variability in cell survival that accompanies harvesting and plating CGN cultures derived from different litters in separate preparations. By concentrating on ethanol-induced cell loss rather on the viable cells counts, far fewer animal litters are necessary to ascertain neuroprotective effects. To determine statistical differences in ethanol-induced cell loss as graphed in the figures, one-way or two-way ANOVAs followed by Tukey post-hoc tests ($p < 0.05$) were used as indicated.

Determinations of $[Ca^{2+}]_i$ from masked ratio image pairs were done by PTI ImageMaster software using the following equation (Grynkiewicz et al. 1985):

$$[Ca^{2+}]_i = \frac{S_{f2}}{S_{b2}} \times (K_D) \times \frac{(R - R_{min})}{(R_{max} - R)}$$

where R is the ratio of fura-2 fluorescence intensity attained at 340 nm over that at 380 nm; K_D is the Fura-2 dissociation constant (224 nM); R_{min} and R_{max} are the intensity ratios correspondingly achieved with maximally free (no calcium) and bound Fura-2 (saturating calcium); and S_{f2} and S_{b2} are the intensities reached at 380 nm excitation for solutions with maximally free and bound Fura-2, respectively.

For calibration, the ratio and intensity values for maximally calcium-bound Fura-2 were obtained using 10 μ M ionomycin to generate ceiling $[Ca^{2+}]_i$ levels. Similarly, ratio and intensity values for maximally unbound Fura-2 were generated using 10 mM EGTA to chelate Ca^{2+} . Empty PDL-treated coverslips with HBSS were used to generate background values. Statistical differences were determined by one-way ANOVA followed by Tukey post-hoc tests ($p < 0.05$) of the maximum values $[Ca^{2+}]_i$ value (peak $[Ca^{2+}]_i$) attained in 5 minutes following ethanol or pharmacological agent addition.

All statistical differences were determined using SPSS Statistics.

Results

To avoid confusion, some terminology needs clarification. The term, “ethanol,” refers exclusively to the reagent, ethyl alcohol, as used in our ethanol exposure paradigms. This differs from the generic use of “alcohol” as used in referring to FASD or in alcohol vulnerability or toxicity. “Ethanol-exposed” and “ethanol-free” described CGN cultures that did or did not receive ethanol, respectively. Regardless of receiving ethanol, the term, “no reagent,” describes culture groups without the addition of other pharmacological agents, which consist of DETA-NONOate, 8-Br-cGMP, Rp-8-pCPT-cGMPS, BDNF, or 2-APB.

Stimulation of the NO Pathway in Pretreatment Paradigms

Still Reduces Alcohol Toxicity

In previous studies regarding the neuroprotection provided by the NO pathway (Bonthius et al. 2004, Bonthius et al. 2003, Pantazis et al. 1993, Pantazis et al. 1995, Pantazis et al. 1998), all protective agents were added simultaneously with alcohol in CGN cultures derived from 9 to 10-day old litters and reduced alcohol toxicity. Since in the previous chapter, calcium chelators and IP_3R antagonists reduced ethanol toxicity only when their addition preceded ethanol exposure, we tested here how sGC and PKG

stimulation affected ethanol-induced cell loss in the same experimental pretreatment paradigm. That is the addition of pharmacological agents here occurred 30 minutes prior to ethanol exposure in CGN cultures derived from mouse litters 5 to 7 days old mice.

A two-way repeated measures ANOVA analyzing how ethanol and DETA-NONOate affected surviving cell numbers revealed a significant interaction between ethanol exposure and DETA-NONOate concentration [$F(5,30) = 9.97, p < 0.001$], and an ethanol exposure effect [$F(1,30) = 51.29, p < 0.001$]. This interaction is due to the ability of DETA-NONOate to influence alcohol-induced cell loss. As illustrated in Figure III-1A, follow-up paired t-tests revealed that DETA-NONOate prevented ethanol's depletion of viable CGNs at the higher concentrations (4.0, 20, 100 μM) ($p < 0.005$). This effect is better demonstrated in Figure III-1B where, much like the calcium chelators in the previous chapter, increasing the concentration of the NO donor, DETA-NONOate, reduced ethanol-induced cell loss from $15.6 \pm 12\%$ to $-1.7 \pm 1.6\%$ [one-way ANOVA, $F(5,36) = 10.27, p < 0.001$]. Although negative cell loss (an increase in cell number) appears at the highest dose (100 μM), this is a natural result of experimental variability. Unlike as previously described (Pantazis et al. 1998), DETA-NONOate failed to show a trophic effect [$F(5,30) = 0.470, p = 0.798$]. These analyses are provided in Table III-1 and 2.

Similarly, a two-way repeated measures ANOVA with ethanol exposure and 8-Br-cGMP concentration as factors yielded a main effect of ethanol exposure [$F(1,5) = 43.72, p < 0.001$] and an interaction between alcohol exposure and 8-Br-cGMP concentration [$F(3,15) = 19.90, p < 0.000$] (Figure III-2A). No main effect of 8-Br-cGMP on the number of surviving CGNs was observed [$F(3,15) = 0.692, p = 0.571$]. As before, this interaction is better visualized by displaying ethanol-induced cell loss as in Figure III-2B. Increasing the 8-Br-cGMP concentration significantly reduced ethanol-induced cell loss [$F(3,20) = 9.2, p < 0.001$]. 8-Br-cGMP (40 μM) reduced ethanol-induced cell loss from $21.0 \pm 2.9\%$ to $4.1 \pm 0.9\%$. These analyses are provided in Tables

III-3 and 4. Concentrations of 8-Br-cGMP greater than 40 μ M were observed in preliminary experiments to be toxic and therefore were not used in this study.

In addition to activating PKG, 8-Br-cGMP is also a potent agonist for cGMP-dependent ion channels (Wei *et al.* 1998, Wei *et al.* 1996), for cGMP phosphodiesterases (Zimmerman *et al.* 1985), and a poor activator of type I (Worner *et al.* 2007) and II cAMP-dependent protein kinases, all of which are expressed in the cerebellum (Kingston *et al.* 1999, Gundlach & Urosevic 1989, Shimizu-Albergine *et al.* 2003, Seeger *et al.* 2003). Therefore to isolate the neuroprotective effect of 8-Br-cGMP to PKG activation, we examined 8-Br-cGMP's ability to rescue CGN cultures from alcohol toxicity following PKG inhibition by Rp-8-pCPT-cGMPS (125 nM). Since Rp-8-pCPT-cGMPS activates cGMP-dependent ion channels while inhibiting PKG (Kramer & Tibbs 1996), Rp-8-pCPT-cGMPS addition prior to 8-Br-cGMP activation can discriminate kinase from ion channel and phosphodiesterase effects.

Figure III-3 shows that Rp-8-pCPT-cGMPS prevents the neuroprotection afforded by 8-Br-cGMP. A 30 minute pretreatment with 8-Br-cGMP significantly reduced the neuronal death generated by 24-hour alcohol exposure (400 mg/dl ethanol) in CGN cultures from $24.1 \pm 2.3\%$ to $-3.9 \pm 3.5\%$ [$t(20) = 6.62, p < 0.001$]. Inhibition of PKG blocks this neuroprotective effect, indicating that 8-Br-cGMP neuroprotection is mediated by PKG. A two-way ANOVA yielded the main effects of PKG inhibition [$F(2,45) = 8.936, p = 0.001$], of 8-Br-cGMP pretreatment [$F(1,45) = 4.760, p = 0.034$], and the interaction of 8-Br-cGMP with Rp-8-pCPT-cGMPS on ethanol-induced cell loss [$F(2,45) = 13.973, p < 0.001$]. This analysis is provided in Table III-5.

8-Br-cGMP Eliminates the Ethanol-Induced Rise in $[Ca^{2+}]_i$

Having established that the neuroprotective effect of 8-Br-cGMP is mediated through PKG, we then tested how 8-Br-cGMP affects alcohol-induced Ca^{2+} elevations. The experiment described in Figure III-4 reveals that 8-Br-cGMP blocks the ethanol-

induced rise in $[Ca^{2+}]_i$ in CGN cultures. Thirty minutes prior to ethanol exposure (400 mg/dl), CGN were pretreated with one of the following: 8-Br-cGMP, 2-APB (an inhibitor of IP_3R), or HBSS. Since we have previously demonstrated in Chapter II that IP_3R blockade by 2-APB pretreatment prevents the alcohol-induced $[Ca^{2+}]_i$ elevations, 2-APB here provided a positive control for inhibition of the alcohol-induced $[Ca^{2+}]_i$ elevations. We have also observed that PKG inhibition does not abrogate the protective effect of 2-APB (data not shown). Figure III-4A shows the field-of-view from representative coverslips from each the three conditions. Prior to ethanol exposure (panels a - c), there are few CGNs with high $[Ca^{2+}]_i$ levels, being mostly blue-green with very few red-colored on the pseudocolor scale. When the same neurons are viewed after ethanol addition, it is obvious that the number of red-shaded neurons increased substantially in the HBSS pretreatment condition (panel d). In contrast, the groups pretreated with 8-Br-cGMP or 2-APB showed little or no increase in the numbers of red-shaded neurons at one minute after ethanol addition (panels e and f).

In Figure III-4B are traces representing the mean $[Ca^{2+}]_i$ levels for each cell population tested following alcohol exposure. In the absence of 8-Br-cGMP or 2-APB, ethanol induced a rapid rise in $[Ca^{2+}]_i$ reaching maximum levels within 30 seconds. One-way ANOVA analysis of the maximum $[Ca^{2+}]_i$ levels attained for each CGN within the recording period shows that pretreatment with 8-Br-cGMP or 2-APB eliminated this $[Ca^{2+}]_i$ rise [$F(2,1365) = 28.38, p < 0.001$]. Like 2-APB (157.2 ± 4.1 nM), 8-Br-cGMP pretreatment reduced the peak $[Ca^{2+}]_i$ attained from 246.0 ± 14.5 nM to 157.0 ± 3.9 nM (Figure III-4C).

Prior or Concurrent 8-Br-cGMP Addition Blocks Ethanol-Induced Toxicity and $[Ca^{2+}]_i$ Elevations

In the present study, we added 8-Br-cGMP 30 minutes before ethanol exposure, yet in previous published studies using CGN cultures, the agents that influence the NO

pathway were added along with ethanol (Bonthius et al. 2004, Bonthius et al. 2003, Pantazis et al. 1995, Pantazis et al. 1998). Although we verified here that prior PKG activation by 8-Br-cGMP provides neuroprotection and eliminates the alcohol-induced $[Ca^{2+}]_i$, no direct comparison on alcohol-induced cell loss was made between pretreatment and simultaneous treatment paradigms. Like the pharmacological agents tested in the previous chapter, 8-Br-cGMP was also present during the entire length of the alcohol exposure.

Therefore we manipulated temporally the addition of 8-Br-cGMP in relation to the alcohol exposure (Figure III-5) as we did in the last chapter with the Ca^{2+} chelators and IP_3R antagonists (Figures II-12 and II-13). When added 30 minutes before or simultaneously with ethanol, 8-Br-cGMP reduced ethanol-induced percent cell loss from 25.5 ± 3.3 to $-3.6 \pm 3.6\%$ and 21.0 ± 4.9 to $-13.4 \pm 4.9\%$, respectively. When added after ethanol exposure, cell death remained at $24.9 \pm 3.1\%$. Ethanol-induced cell loss was subjected to a 2x3 two-way ANOVA with 8-Br-cGMP concentration (0 or 40 μM) and 8-Br-cGMP treatment time in relation to alcohol exposure (30 min before, 0 min, or 30 min after) as independent measures. As evident in the figure, this analysis yielded an interaction of 8-Br-cGMP treatment time with 8-Br-cGMP concentration [$F(2,30) = 9.9$, $p = 0.001$], which is the result of 8-Br-cGMP's inability to rescue CGNs from alcohol toxicity. Therefore 8-Br-cGMP must be added either before or at the same time, but not after, alcohol exposure is initiated in order to obtain a neuroprotective effect. This analysis is provided in Table III-6.

Since the experiment described in Figure III-5, demonstrated that prior or simultaneous 8-Br-cGMP addition with ethanol ameliorates alcohol-induced cell death, we wondered if simultaneous 8-Br-cGMP addition with ethanol also blocks the alcohol-induced $[Ca^{2+}]_i$ elevation. In Figure III-6, we manipulated temporally the addition of 8-Br-cGMP in relation to the alcohol exposure while measuring peak $[Ca^{2+}]_i$ levels. Fura-2-loaded CGN cultures received either 8-Br-cGMP (40 μM) or PBS, 30 minutes before

imaging. After determining baseline $[Ca^{2+}]_i$ values (for 15 seconds), one of the following additions were made at time = 0 seconds: PBS, 8-Br-cGMP (40 μ M), ethanol (400 mg/dl), or 8-Br-cGMP with ethanol. A one-way ANOVA of the peak $[Ca^{2+}]_i$ levels across treatment conditions yielded the main effect of treatment condition [$F(4,1827) = 43.7, p < 0.001$]. A post-hoc Tukey test revealed that the ethanol-alone treatment condition differed from all other groups ($p < 0.05$). Here, in the absence of other pharmacological agents, cells attained the peak $[Ca^{2+}]_i$ level of 161.1 ± 5.2 nM from ethanol exposure. As can be seen, the simultaneous addition of 8-Br-cGMP with ethanol prevented the alcohol-induced increase of $[Ca^{2+}]_i$ (mean = 101.6 ± 2.9 nM) which resembled the effect that 8-Br-cGMP pretreatment had on alcohol-induced $[Ca^{2+}]_i$ elevations (115.5 ± 2.6 nM). For comparison, cells receiving PBS and 8-Br-cGMP additions alone (at time = 0 minutes) attained peak $[Ca^{2+}]_i$ levels of 110.6 ± 3.3 and 110.0 ± 3.8 nM, respectively.

Since the neuroprotection afforded by the NO signaling pathway depends on PKG function (Bonthius et al. 2004, Pantazis et al. 1998), we next examined how BDNF, a neuroprotective agent that doesn't rely on PKG (Bonthius et al. 2003), affects the alcohol-induced $[Ca^{2+}]_i$ elevations. As indicated in Figure III-7A, a one-way ANOVA yielded the main effect of treatment condition [$F(3,1115) = 56.0, p < 0.001$]. BDNF pretreatment failed to stop the ethanol-induced $[Ca^{2+}]_i$ elevation which attained a peak level of 114.1 ± 4.5 nM. Although similar in that they both produced $[Ca^{2+}]_i$ elevations, BDNF pretreatment (1 ng/ml) followed by ethanol addition generated significantly higher peak $[Ca^{2+}]_i$ levels than those attained by ethanol addition alone (98.1 ± 3.8 nM) ($p < 0.05$). For comparison, the peak $[Ca^{2+}]_i$ level attained for BDNF pretreatment without ethanol addition was 62.8 ± 1.9 nM. Trace data from BDNF addition while recording resembles the trace containing only a PBS addition (0 mg/dl EtOH) in Figure II-3 (data not shown). This indicates that BDNF addition alone does not elevate $[Ca^{2+}]_i$, as CGN cultures only attained the mean peak $[Ca^{2+}]_i$ levels of only 65.5 ± 2.5 nM.

As this experiment examined the effects of BDNF pretreatment on $[Ca^{2+}]_i$, we also assessed the effects of BDNF pretreatment on alcohol-induced cell loss and found that it was still protective, reducing cell loss from $22.3 \pm 4.5\%$ to $-6.4 \pm 10.5\%$ as shown in Figure 7B [$t(7) = 2.51$, $p = 0.027$].

Discussion

Within the developing cerebellum, activation of glutamate NMDA receptors provide an important neurotrophic stimulus (Balazs et al. 1988, Balazs et al. 1989, Burgoyne et al. 1993) that relies on NO signaling (Pantazis et al. 1998). Glutamate NMDA receptors allow Ca^{2+} into developing neurons which then activates Ca^{2+} -dependent nNOS (Bredt & Snyder 1989, Southam et al. 1991) to generate NO (Abbott & Nahm 2004). The NO generated by NOS, stimulates sGC to convert GTP into cGMP (Moncada & Higgs 1995). By binding to the regulatory subunits of PKG, cGMP activates PKG, thus allowing it to phosphorylate multiple substrates at serine/threonine sites (Francis & Corbin 1999).

Through this same mechanism via the NO signaling pathway, NMDA activation also moderates alcohol neurotoxicity (Pantazis et al. 1998). In CGN cultures, NO mimics NMDA in reducing alcohol-induced cell loss. Blocking nNOS, sGC, or PKG downstream abolishes this neuroprotective effect, indicating that NMDA-mediated neuroprotection to alcohol-induced cell loss depends on NO signaling (Pantazis et al. 1998, Bonthius et al. 2004). CGN cultures derived from mice with a null mutation for nNOS ($nNOS^{-/-}$) are also more susceptible to alcohol toxicity than cultures originating from wild-type mice in that alcohol always precipitates significantly greater cell death (Bonthius et al. 2002). Similarly, $nNOS^{-/-}$ mice also exhibit greater alcohol-induced cerebellar and hippocampal damage than wild-type mice and also fail to develop any alcohol resistance, thus demonstrating their greater vulnerability to alcohol toxicity (Bonthius et al. 2002, Bonthius et al. 2006).

This study focused on identifying downstream mechanisms through which PKG can transmit the neuroprotective signal provided by the activation of NO signaling pathway. The evidence presented in Chapter II indicated that alcohol elevates $[Ca^{2+}]_i$ to precipitate cell death in primary neuronal cultures, and supports the *in vivo* observations in mouse and avian embryos that alcohol exposure triggers apoptosis (Kilburn *et al.* 2006, Debelak-Kragtorp *et al.* 2003). Incidentally, PKG can also regulate the intracellular release of calcium (Felbel *et al.* 1988, Haug *et al.* 1999, Schlossmann *et al.* 2000). Under the context of FASD, the present study links PKG-mediated neuroprotection to blocking alcohol-induced $[Ca^{2+}]_i$ elevations, which is a key event in alcohol-induced cell death.

PKG Activation Reduces Alcohol-Induced Neuronal Death and $[Ca^{2+}]_i$ Elevations

This study dismisses cGMP-dependent ion channels (Wei *et al.* 1998, Wei *et al.* 1996) and cGMP phosphodiesterases (Zimmerman *et al.* 1985) as part of the NO signaling pathway that shields CGN cultures from alcohol toxicity. Like in previous studies (Bonthius *et al.* 2004, Pantazis *et al.* 1998), we show that both the NO generator, DETA-NONOate, and PKG activator, 8-Br-cGMP, reduced alcohol's depletion of CGN cultures. Co-addition of PKG inhibitor, Rp-8-pCPT-cGMPS, with 8-Br-cGMP prevents PKG stimulation while allowing 8-Br-cGMP to activate cGMP-dependent ion channels (Wei *et al.* 1998, Wei *et al.* 1996) and cGMP phosphodiesterases (Zimmerman *et al.* 1985). Since co-addition of Rp-8-pCPT-cGMPS with 8-Br-cGMP eliminated 8-Br-cGMP's neuroprotective effects, this indicates that the neuroprotective pathway requires PKG. These results are consistent with previous findings in CGN cultures that also identify PKG as an essential component for the neuroprotection provided by the NO signaling pathway (Bonthius *et al.* 2004). In that study (Bonthius *et al.* 2004), PKG stimulation by 8-Br-cGMP restores the neuroprotection provided by NMDA and DETA-

NONOate and lost through sGC inhibition by LY83583. PKG inhibitor, Rp-8-pCPT-cGMPS, also blocks the neuroprotective effects of NGF, FGF, and IGF; neurotrophins that utilize the NO signaling pathway (Bonthius et al. 2003).

One important difference that we observed here from earlier studies was that DETA-NONOate and 8-Br-cGMP failed to enhance survival in ethanol-free cultures. That is, the cell numbers in ethanol-free cultures receiving either agent did not increase in comparison to reagent-free cultures. This observation is not due to cell proliferation since previous cell cycle analyses have established that our CGN cultures do not proliferate (Pantazis et al. 1993). A possible explanation for this phenomenon may lie in the age of neuronal source for our CGN cultures. In this study, CGN cultures were harvested from 5 to 7 day old litters, whereas the neuronal cells used in previous studies originated from 9 to 10 day old pups (Bonthius et al. 2004, Bonthius et al. 2003, Pantazis et al. 1993, Pantazis et al. 1995, Pantazis et al. 1998).

PKG Eliminates Alcohol-Induced $[Ca^{2+}]_i$ Elevations to Prevent Alcohol Toxicity

Having established that the neuroprotective effect of 8-Br-cGMP is mediated through PKG, we focused on putative downstream mechanisms through which PKG transmits its neuroprotective effect. PKG can regulate the Ca^{2+} release from intracellular stores by phosphorylating and inhibiting IP_3Rs located on ER membranes (Felbel et al. 1988, Haug et al. 1999, Schlossmann et al. 2000). Coincidentally, alcohol elevates $[Ca^{2+}]_i$ through IP_3R -dependent release of Ca^{2+} from intracellular stores, an event connected to cell death as observed in primary neuronal cultures in Chapter II, and in mouse and avian embryos (Kilburn et al. 2006, Debelak-Kragtorp et al. 2003).

This study links PKG-mediated neuroprotection to a reduction of alcohol's ability to elevate $[Ca^{2+}]_i$. As we observed, PKG stimulation by 8-Br-cGMP completely eliminated alcohol-induced $[Ca^{2+}]_i$ elevations in CGN cultures, mimicking the inhibition

provided by IP₃R blockade through 2-APB addition. The neuroprotective effect of 8-Br-cGMP was lost when it was added after initiating alcohol exposure. Therefore 8-Br-cGMP must be added either before or at the same time, but not after, alcohol exposure is initiated in order to ameliorate alcohol toxicity. Prior or concurrent 8-Br-cGMP addition to CGN cultures also abolished alcohol-induced elevations of [Ca²⁺]_i. Since alcohol induces an increase in [Ca²⁺]_i within seconds, it is unlikely that the addition of 8-Br-cGMP 30 minutes after initiating alcohol exposure could modulate alcohol's rapid effect on [Ca²⁺]_i. These results suggest that 8-Br-cGMP is an effective neuroprotective agent only when it has the opportunity to block the rapid alcohol-induced increase in [Ca²⁺]_i, supporting the hypothesis that the alcohol-induced increase in [Ca²⁺]_i promotes cell death.

Although the evidence presented in chapter II indicates that eliminating alcohol-induced [Ca²⁺]_i elevations is neuroprotective, moderating alcohol toxicity does not necessitate reducing [Ca²⁺]_i levels since BDNF, a non-PKG-dependant neuroprotective agent (Bonthius et al. 2003), does not block alcohol-induced [Ca²⁺]_i elevations. Although the neuroprotective effects of PKG and BDNF against alcohol-induced neuronal death are similar, their mechanisms of action are quite different. BDNF binds to p75 and to TrkB receptors (Binder & Scharfman 2004) both of which are expressed in CGN cultures (Koshimizu *et al.* 2010, Lindholm *et al.* 1993). Other studies have indicated that BDNF relies on a phosphoinositide 3-kinase-dependent pathway to achieve neuroprotection (Nonomura *et al.* 1996, Shimoke *et al.* 1997, Leeds *et al.* 2005).

Mechanisms Underlying PKG-Mediated Inhibition of the Alcohol-Induced [Ca²⁺]_i Elevations

Identified in this study is a downstream effector through which the NO signaling pathway via PKG may diminish alcohol toxicity: the reduction of alcohol-induced [Ca²⁺]_i elevations. One likely mechanism that PKG may regulate [Ca²⁺]_i levels is through modulating IP₃R activity. Since IP₃Rs contain PKG phosphorylation sites (Komalavilas

& Lincoln 1994, Haug et al. 1999) and comprise a major conduit for Ca^{2+} release from intracellular stores (Furuichi & Mikoshiba 1995), it is likely that IP_3R phosphorylation by PKG constitutes an additional regulatory mechanism in Ca^{2+} release from the ER. IP_3Rs may also represent a target for NO-induced ethanol resistance, being both strategically located and functionally downstream of PKG. A physiological role for NO-mediated control of IP_3Rs in mediating ethanol resistance is also supported in that IP_3Rs are heavily expressed in ethanol-susceptible Purkinje neurons (Ross et al. 1992).

IP_3R phosphorylation by PKG was first described using tissues derived from aortic smooth muscle (Komalavilas & Lincoln 1994, Koga et al. 1994, Komalavilas & Lincoln 1996), platelets (el-Daher et al. 1996), and cerebellum (Koga et al. 1994, Haug et al. 1999). PKG phosphorylates two serines within IP_3R (Haug et al. 1999). Inhibition of agonist-induced intracellular Ca^{2+} currents by PKG has been described in a variety of cell systems including smooth muscle (Murthy & Makhlof 1995, Murthy et al. 1993, Murthy & Zhou 2003), megakaryocytes (Tertyshnikova et al. 1998), and more recently in neural tissue (Fernandez et al. 2005, Morales et al. 2005). These findings suggest that PKG activity attenuates IP_3R Ca^{2+} conductance by phosphorylation.

Interestingly, PKG potentiation of IP_3R -mediated intracellular Ca^{2+} currents has also been observed in hepatocytes (Guihard et al. 1996, Rooney et al. 1996). One study (Wagner et al. 2003), in order to investigate the effects of IP_3R phosphorylation in a null background, transfected cell lines devoid of endogenous IP_3R with IP_3 receptor, type I ($\text{IP}_3\text{R1}$). Selective mutation of these phosphorylation sites in $\text{IP}_3\text{R1}$ abolished PKG-induced Ca^{2+} currents (Wagner et al. 2003, Wagner et al. 2004). These findings appear to conflict with the notion that PKG phosphorylation universally inhibits IP_3R function.

A rationale for this discrepancy may involve the IP_3R isoforms under study. Presently, three isoforms of IP_3Rs have been cloned, denoted as types I, II, and III (Taylor et al. 1999). The $\text{IP}_3\text{R1}$ isoform undergoes alternative splicing, allowing for long and short forms (Danoff et al. 1991). The long form is the predominant splice variant

found in adult neuronal tissues, whereas the short form is mainly expressed in fetal brain and peripheral tissues (Danoff et al. 1991). Unlike the long form of the IP₃R1 (Nakade et al. 1994), phosphorylation of the short form reduces IP₃-induced Ca²⁺ release (Tertyshnikova & Fein 1998, Tertyshnikova et al. 1998). Therefore, functional different tissue specific splice variants may explain the observed inconsistencies in the effects of IP₃R1 phosphorylation by PKG. Studies that found phosphorylation-induced inhibition of IP₃R activity likely dealt with the short form of the IP₃R1.

Another plausible explanation for this discrepancy links NO modulation of IP₃R function to expression of IP₃ receptor-associated cGMP kinase substrate (IRAG), also found on ER membranes (Schlossmann et al. 2000). IRAG forms functional complexes with PKG-I β and IP₃R1 to decrease Ca²⁺ currents (Schlossmann et al. 2000), but not with PKG-I α or PKG-II (Ammendola et al. 2001). Since IRAG mRNA antisense oligonucleotides block NO inhibition of IP₃R1-mediated Ca²⁺ release, this suggests that IRAG expression is a precondition at least for PKG-I β and IP₃R1 interactions (Fritsch et al. 2004, Geiselhoringer et al. 2004b).

For IRAG to confer ethanol-resistance, IRAG must inhibit ER-mitochondrial Ca²⁺ signaling by complexing with the PKG and IP₃R isoforms available in vulnerable brain regions. IP₃R1, IP₃R3, but not IP₃ receptor, type II, (IP₃R2) cerebral distribution (Sharp et al. 1999, Furuichi et al. 1990, Newton et al. 1994) mirrors the regions of ethanol-susceptibility, showing high cerebellar and hippocampal expression. Since central nervous system IP₃R2 expression is limited to spinal cord glia (Newton et al. 1994, Sharp et al. 1999), it cannot be responsible for ethanol-induced apoptosis. In adult mice, PKG-I β expression is high in the hippocampus and olfactory bulb, but low in the cerebellum (Geiselhoringer et al. 2004a). Since IRAG's cerebral distribution in adult mice is limited to the thalamus (Geiselhoringer et al. 2004a) and does not co-localize with PKG-I β in the same brain regions, these data suggest that NO-mediated ethanol resistance may not involve IRAG.

Conclusions and Future Directions

In summary, this study demonstrates that activating the NO signaling pathway downstream at PKG with 8-Br-cGMP rescues primary neuronal cell cultures from alcohol toxicity. We observed that cGMP-mediated neuroprotection occurs through PKG activation, and that PKG activation blocks alcohol-induced $[Ca^{2+}]_i$ elevations, an effect that resembles IP₃R inhibition. Altering the time of alcohol exposure in relation to 8-Br-cGMP addition, established that PKG's reduction of alcohol toxicity depends on preventing alcohol-induced $[Ca^{2+}]_i$ elevations. These findings support our results in Chapter II: preventing the alcohol-induced $[Ca^{2+}]_i$ elevation rescues neuronal cultures. One likely mechanism by which PKG can influence $[Ca^{2+}]_i$ is by phosphorylating and consequently inhibiting the IP₃R.

BDNF, which does not rely on PKG to provide neuroprotection, failed to block alcohol-induced $[Ca^{2+}]_i$ elevations while preventing alcohol toxicity. This indicates that averting the alcohol-induced $[Ca^{2+}]_i$ elevations is not a necessary requirement of neuroprotection. BDNF may prevent cell death by ameliorating a compromised $[Ca^{2+}]_i$ -dependent process downstream from the alcohol-induced disruption of $[Ca^{2+}]_i$. Another possibility is that alcohol disturbs multiple cellular processes to precipitate neuronal death. Avoiding the disruption of a single process, such as $[Ca^{2+}]_i$ via PKG activation, is sufficient to rescue the neuron. BDNF may interact with a different as yet unidentified cellular process, preventing its disruption by alcohol and subsequent cell death.

Figure III-1. NO donor, DETA-NONOate, rescues CGN cultures from ethanol-induced neuronal death. The bars in panel A represent mean \pm SEM cell numbers of viable CGNs for each condition as estimated by trypan blue exclusion for 7 litters. * indicates significance as determined by follow-up paired t-tests comparing ethanol-free and ethanol-exposed conditions. For panel B, ethanol-induced percent cell loss was calculated by comparing ethanol-exposed groups to ethanol-free cultures similar pretreated with DETA-NONOate. Error bars represent \pm SEM. Means without a shared letter differ significantly as determined by one-way ANOVA and Tukey post-hoc tests ($p < 0.05$).

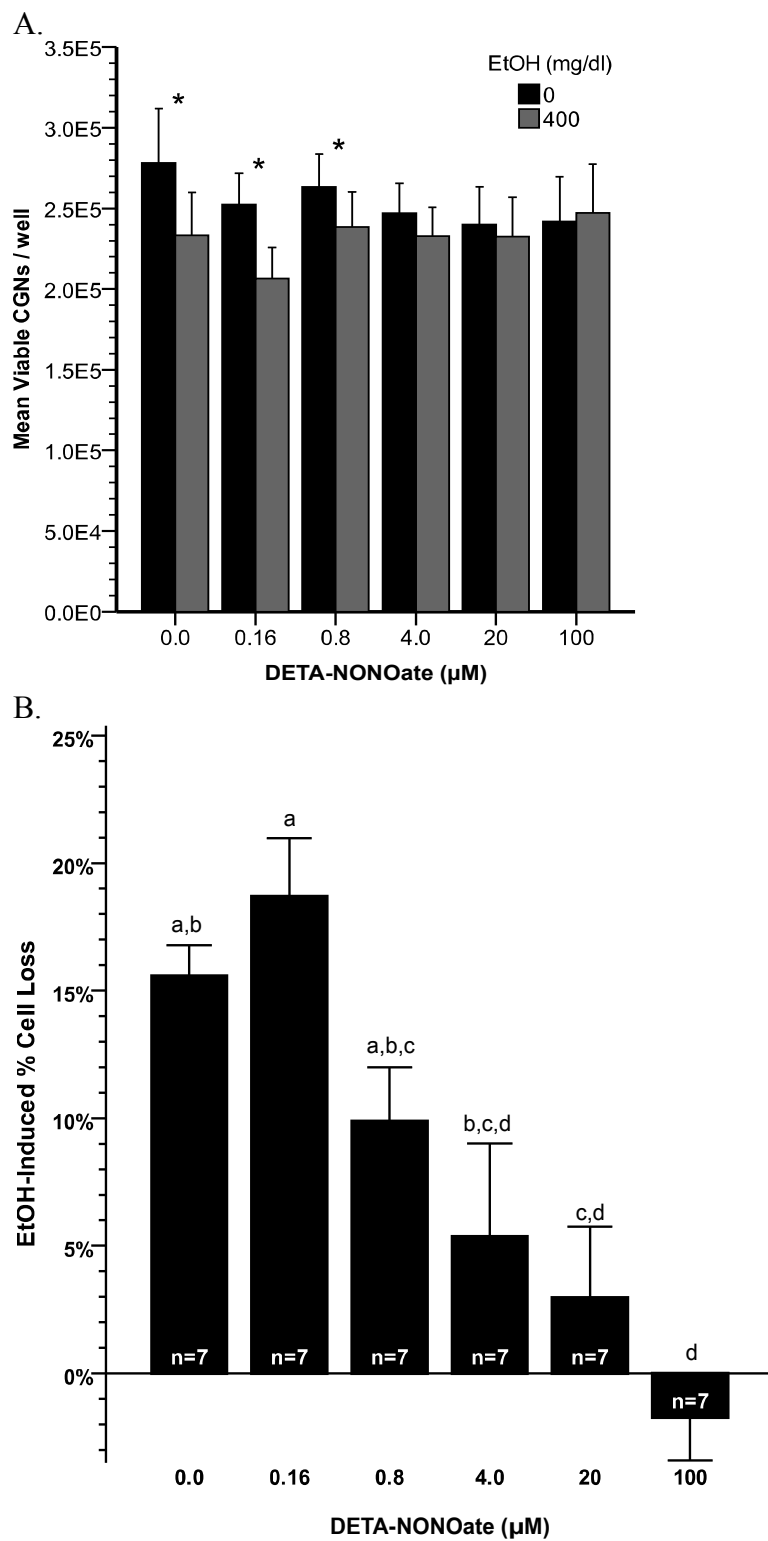


Table III-1. Two-Way Repeated Measures ANOVA for the effect of ethanol and DETA-NONOate on surviving CGN numbers.

Source	Sum of Squares	df	Mean Square	<i>F</i>	<i>p</i>
EtOH	1.01E+10	1	1.01E+10	51.29	0.000
Error	1.18E+09	6	1.97E+08		
DETA-NONOate	6.47E+09	5	1.29E+09	0.47	0.798
Error	8.31E+10	30	2.77E+09		
EtOH * DETA-NONOate	7.44E+09	5	1.49E+09	9.97	0.000
Error	4.48E+09	30	1.49E+08		

Table III-2. One-Way ANOVA for the effect of DETA-NONOate on ethanol-induced cell loss.

	Sum of Squares	df	Mean Square	<i>F</i>	<i>p</i>
Between Groups	2106.15	5	421.231	10.27	0.000
Within Groups	1476.92	36	41.026		
Total	3583.07	41			

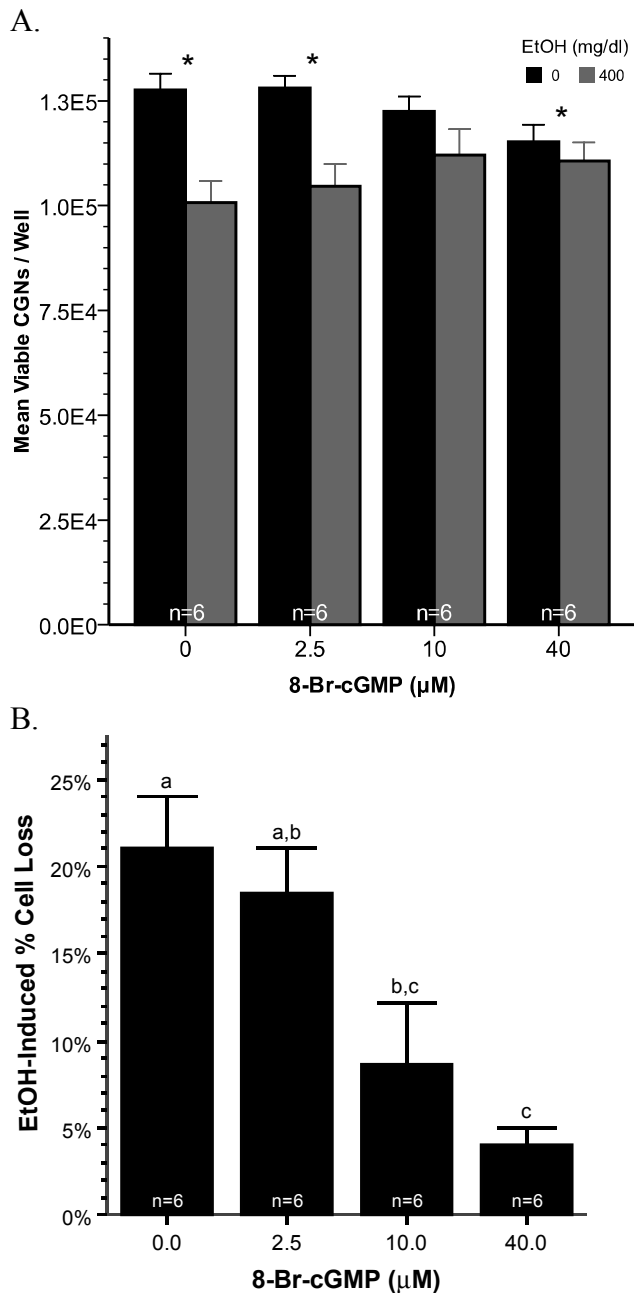


Figure III-2. PKG activation reduces ethanol-induced neuronal death. Axes and scripts (*, a, b, or c) are explained in Figure III-1. Panel A shows viable CGN numbers with and without the presence of ethanol (400 mg/dl) and 8-Br-cGMP. Panel B illustrates that pretreatment with PKG-activator, 8-Br-cGMP, produced a dose-dependent reduction in ethanol-toxicity.

Table III-3. Two-Way repeated measures ANOVA for the effect of ethanol and 8-Br-cGMP on surviving CGN numbers.

Source	Sum of Squares	df	Mean Square	<i>F</i>	<i>p</i>
EtOH	3.19E+09	1	3.19E+09	43.72	0.001
Error	3.65E+08	5	7.30E+07		
8-Br-cGMP	1.40E+08	3	4.67E+07	0.69	0.571
Error	1.01E+09	15	6.74E+07		
EtOH * 8-Br-cGMP	9.96E+08	3	3.32E+08	19.90	0.000
Error	2.50E+08	15	1.67E+07		

Table III-4. One-Way ANOVA for the effect of 8-Br-cGMP on ethanol-induced cell loss.

	Sum of Squares	df	Mean Square	<i>F</i>	<i>p</i>
Between Groups	1156.99	3	385.664	9.21	0.000
Within Groups	837.91	20	41.896		
Total	1994.90	23			

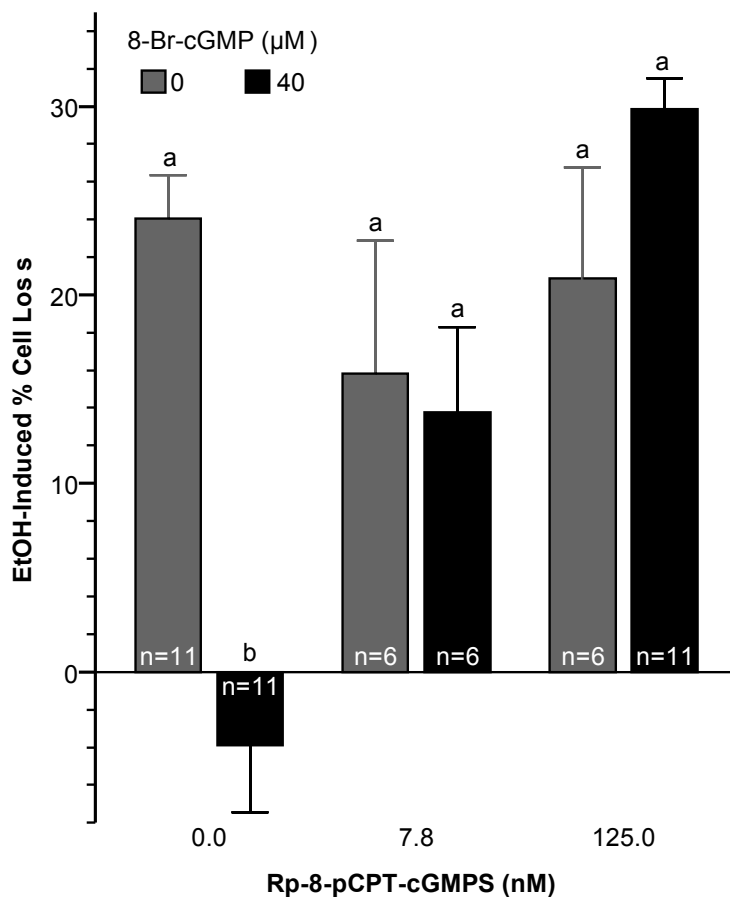


Figure III-3. PKG inhibitor, Rp-8-pCPT-cGMPS, prevents neuroprotection by 8-Br-cGMP. Before overnight alcohol-exposure (400 mg/dl ethanol), CGN cultures were treated with 8-Br-cGMP or PKG inhibitor, Rp-8-pCPT-cGMPS, as indicated on the x axes. The bar color (black versus grey) indicates the presence of 8-Br-cGMP. Bars and letters (a, b) are explained in Figure III-1. 8-Br-cGMP pretreatment reduced alcohol-induced cell death, whereas Rp-8-pCPT-cGMPS blocks this neuroprotective effect, indicating that 8-Br-cGMP neuroprotection is mediated by PKG.

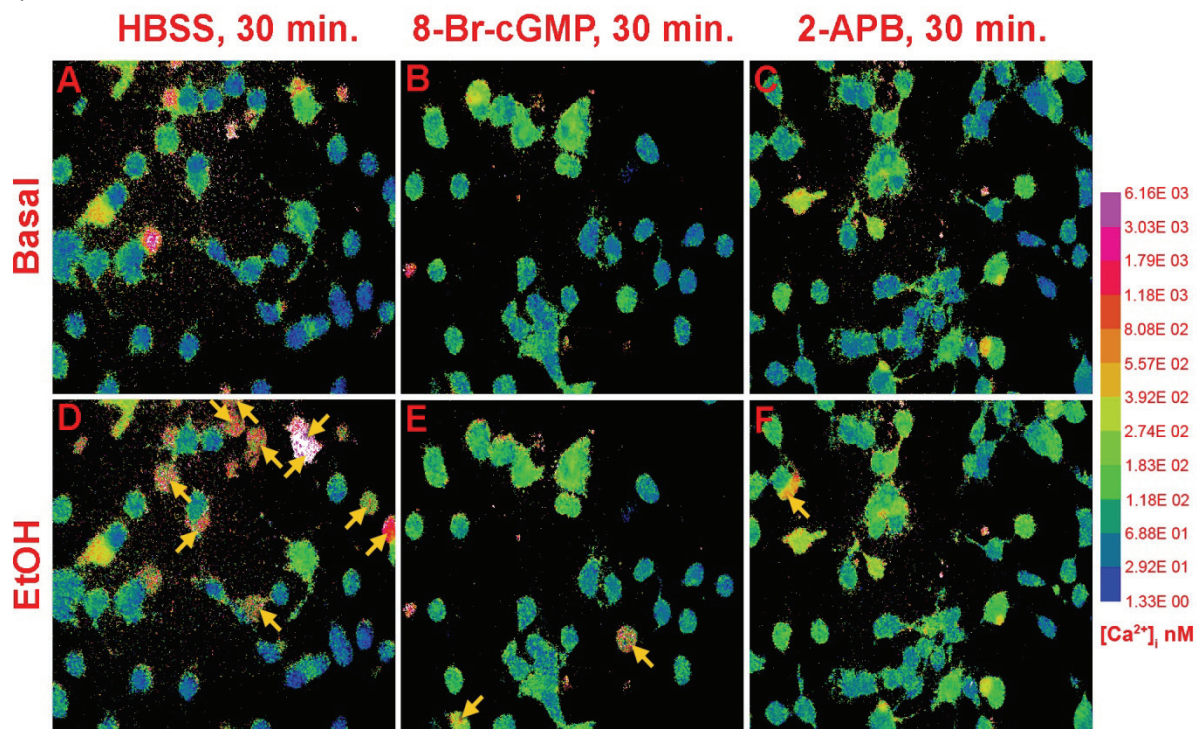
Table III-5. Two-Way ANOVA for the effect of 8-Br-cGMP and Rp-8-pCPT-cGMPS on ethanol-induced cell loss.

	Sum of Squares	df	Mean Square	<i>F</i>	<i>p</i>
8-Br-cGMP	572.67	1	572.67	4.76	0.034
Rp-8-pCPT-cGMPS	2150.16	2	1075.08	8.94	0.001
8-Br-cGMP * Rp-8-pCPT-cGMPS	3362.09	2	1681.05	13.97	0.000
Error	5413.69	45	120.30		

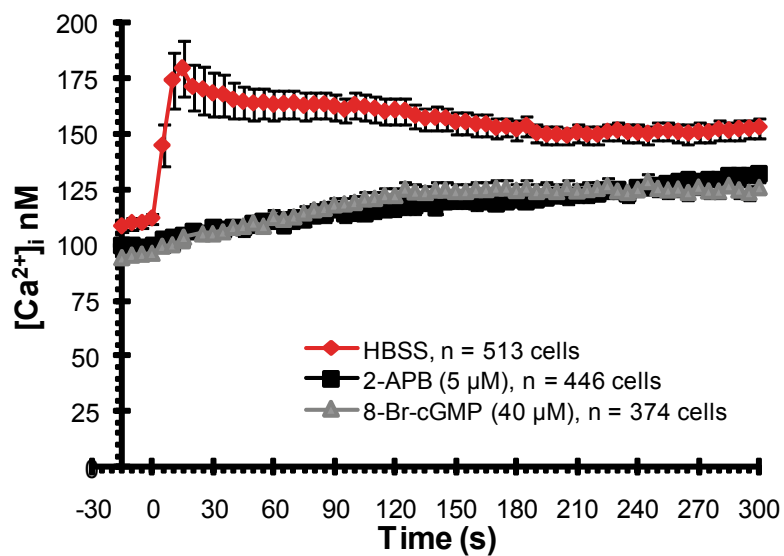
Figure III-4. Pretreatment with either 8-Br-cGMP or 2-APB blocks the rapid ethanol-induced rise in $[Ca^{2+}]_i$ in CGN cultures. Figure III-3A shows pseudocolor 340/380 ratio images of Fura-2 fluorescence in CGN cultures. Panels a – c depict fields from each of the three pretreatment groups [HBSS, 8-Br-cGMP (40 μ M), 2-APB (5 μ M), respectively] before alcohol exposure (800 mg/dl), whereas panels d – f are the identical fields but viewed approximately one minute after initiating ethanol exposure. The colored scale bar on the right maps $[Ca^{2+}]_i$ levels with blue and red depicting low and high $[Ca^{2+}]_i$, respectively. The arrows in lower panels identify individual neurons that show substantial ethanol-induced increases in $[Ca^{2+}]_i$ as compared with the panel above.

Figure III-4B shows $[Ca^{2+}]_i$ traces from CGN cultures that were pretreated with 8-Br-cGMP (40 μ M), 2-APB (5 μ M), or HBSS (control) for 30 minutes prior to initiating ethanol exposure. Intracellular Ca^{2+} was recorded for 5 minutes following ethanol addition (400 mg/dl). In the same cell cultures, the bars in Figure III-4C indicate the mean \pm SEM of the peak $[Ca^{2+}]_i$ attained for all cells in each treatment condition across 4 mouse litters. The peak $[Ca^{2+}]_i$ level for every cell corresponds to the highest $[Ca^{2+}]_i$ value attained over the 5 minutes following ethanol addition. The dashed line indicates the baseline $[Ca^{2+}]_i$ level, which was calculated as the mean unstimulated $[Ca^{2+}]_i$ for all the cells in this experiment. Unstimulated basal $[Ca^{2+}]_i$ levels for each cell were calculated by averaging the $[Ca^{2+}]_i$ measured in the 15 seconds preceding alcohol exposure. Means without a shared letter differ significantly as determined by Tukey post-hoc tests ($p < 0.05$).

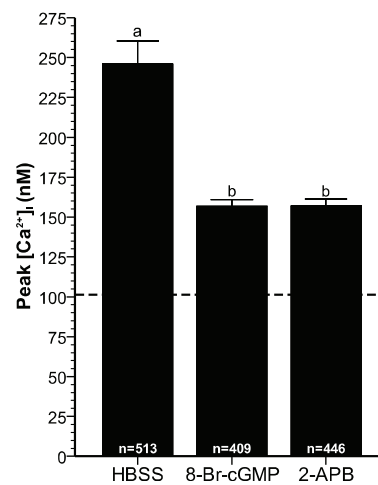
A.



B.



C.



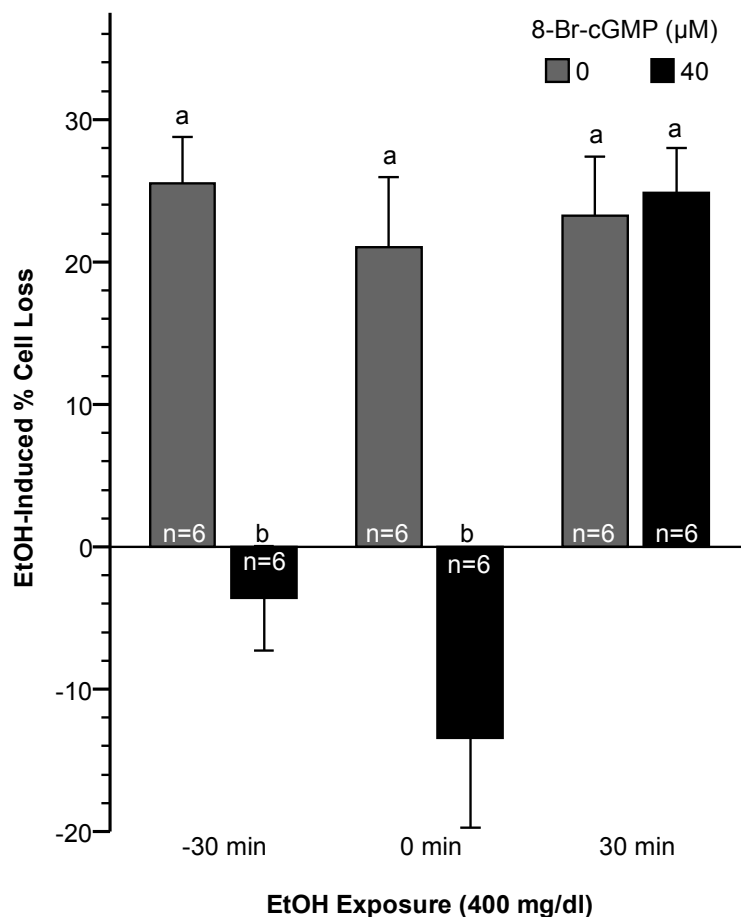


Figure III-5. The neuroprotective effect of 8-Br-cGMP is lost when it is added after initiating ethanol exposure. This figure shows ethanol-induced percent cell loss when CGNs are treated with 8-Br-cGMP (0 or 40 μM) either 30 minutes before, simultaneously with, or 30 minutes after ethanol exposure (400 mg/dl) has begun. Consistent with previous studies, 8-Br-cGMP was neuroprotective when added 30 minutes before and simultaneously with ethanol. When added 30 minutes after ethanol alcohol exposure has begun, the protective effect is lost. The letters (a, b) are explained in Figure III-1.

Table III-6. Two-Way ANOVA for the effect of 8-Br-cGMP and Treatment Time on ethanol-induced cell loss.

	Sum of Squares	df	Mean Square	<i>F</i>	<i>p</i>
Treatment Time	2535.25	2	1267.63	11.05	0.000
8-Br-cGMP	3838.03	1	3838.03	33.45	0.000
Treatment Time * 8-Br-cGMP	2270.97	2	1135.49	9.90	0.001
Error	3442.24	30	114.74		

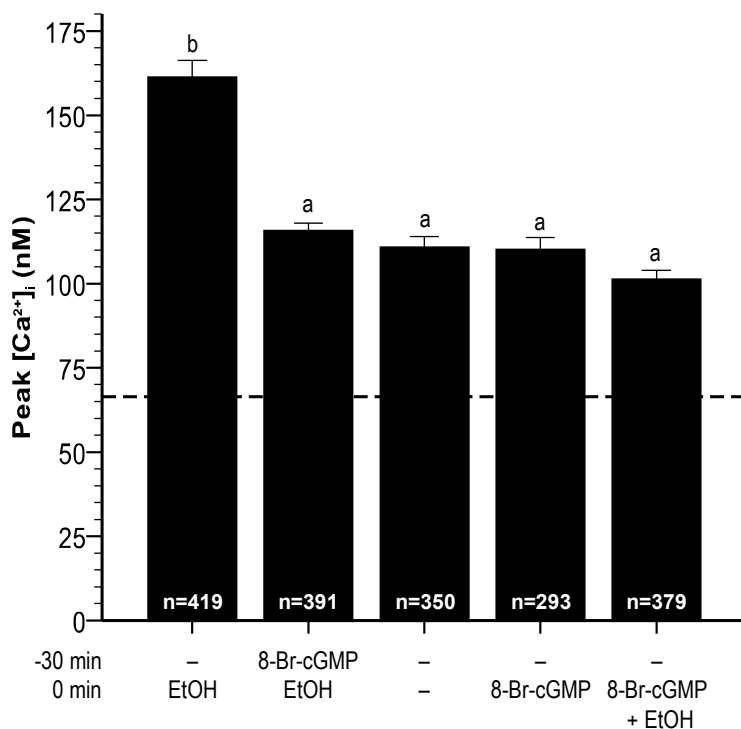


Figure III-6. Concurrent addition of 8-Br-cGMP with ethanol prevents alcohol-induced $[Ca^{2+}]_i$ elevations. For each treatment condition, the bars (mean \pm SEM) represent the summary data (peak $[Ca^{2+}]_i$) pooled from the cells of 5 mouse litters. For every cell, the peak $[Ca^{2+}]_i$ level corresponds to the highest $[Ca^{2+}]_i$ value attained over the 9 minutes following ethanol addition. The dashed line indicates the baseline $[Ca^{2+}]_i$ level, which was calculated as the mean unstimulated $[Ca^{2+}]_i$ for all the cells in this experiment. Unstimulated basal $[Ca^{2+}]_i$ levels for each cell were calculated by averaging the $[Ca^{2+}]_i$ measured in the 15 seconds preceding alcohol exposure. Statistical differences were determined by one-way ANOVA followed by Tukey post-hoc tests ($p < 0.05$). The letters (a, b) are explained in Figure III-1.

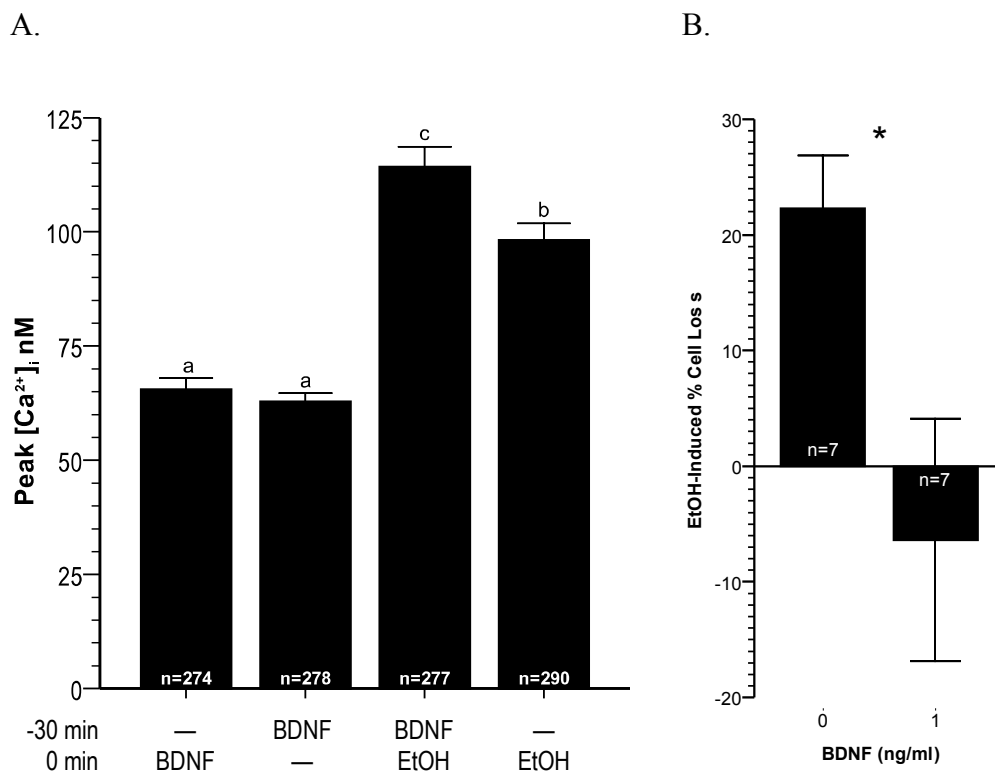


Figure III-7. BDNF, a non-PKG-dependent neuroprotective agent, does not block alcohol-induced $[Ca^{2+}]_i$ elevations. In panel A, the bars (mean \pm SEM) correspond to the peak $[Ca^{2+}]_i$ data combined from 4 mouse litters in the treatment conditions shown on the x axis. The peak $[Ca^{2+}]_i$ level for every cell is the highest $[Ca^{2+}]_i$ value attained over the 5 minutes following ethanol addition (400 mg/dl). Statistical differences were determined by one-way ANOVA followed by Tukey post-hoc tests as denoted by the letters (a, b) ($p < 0.05$). Panel B shows ethanol-induced percent cell loss following BDNF pretreatment (0 or 1 ng/ml) in 7 mouse litters. * indicates significance as determined by an independent samples t-test comparing percent cell loss in BDNF-free and BDNF-treated conditions.

CHAPTER IV

CONCLUDING REMARKS

Summary

In the context of FASD, we investigated how the NO signaling pathway influences intracellular Ca^{2+} release to mediate alcohol resistance using a primary cell culture model consisting of CGN. Alcohol exposure during neurogenesis induces abnormally high apoptotic neuronal death across multiple brain regions, including in the cerebellum (Sowell et al. 1996, O'Hare et al. 2005, Archibald et al. 2001, Goodlett et al. 1990, Maier et al. 1997, Bonthius et al. 2002, Chen et al. 1998). Previous studies established that the NO signaling pathway (NOS \rightarrow NO \rightarrow sGC \rightarrow cGMP \rightarrow PKG) ameliorates alcohol toxicity, consequently diminishing neuronal loss both *in vivo* (Bonthius et al. 2006, Bonthius et al. 2002) and *in vitro* (Bonthius et al. 2004, Bonthius et al. 2003, Pantazis et al. 1995, Pantazis et al. 1998). Endoplasmic reticulum Ca^{2+} release, a key apoptotic mechanism (Duchen 2000, Rao *et al.* 2004), requires the IP_3R , a known PKG substrate (Komalavilas & Lincoln 1994, Haug et al. 1999). The studies outlined in this dissertation exposed a downstream mechanism for how NO signaling can moderate alcohol toxicity and focused on a crucial intersection point where the nitric oxide signaling cascade influences Ca^{2+} -mediated apoptotic mechanisms. In these studies, our central hypothesis has been that while alcohol disturbs Ca^{2+} homeostasis to trigger apoptosis, the NO signaling pathway counteracts this by restraining Ca^{2+} release from the ER.

The experiments outlined in Chapters II examined the relationship between alcohol-induced cell death and intracellular Ca^{2+} . Our results suggested that alcohol increases $[\text{Ca}^{2+}]_i$ and cell loss through abnormal Ca^{2+} release from intracellular stores rather than Ca^{2+} influx from extracellular space. Both inhibitors of ER- Ca^{2+} release and Ca^{2+} chelators abrogated the alcohol-induced $[\text{Ca}^{2+}]_i$ increases and cell loss. These

findings not only verified earlier studies linking alcohol-induced $[Ca^{2+}]_i$ elevations to cell death (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005, Hirata et al. 2006), but also extended them by identifying the source of the Ca^{2+} surge as originating from intracellular stores and by establishing that alcohol, by raising $[Ca^{2+}]_i$ early on, initiates the cell death which occurs hours later. Although the mechanisms by which the alcohol-induced rise in $[Ca^{2+}]_i$ triggers cell death are still under investigation, our evidence suggested that Ca^{2+} initiates multiple parallel apoptotic signaling cascades that synergistically interact to precipitate cell death. Inhibiting either calcineurin or CaMKii, which are found in separate Ca^{2+} -activated pathways, shielded CGN cultures from alcohol toxicity. Blocking the MCU and the MPTP also ameliorated alcohol-induced neuronal loss. That the activated pathways must interact to precipitate cell death likely explains why inhibition of one of many parallel Ca^{2+} -activated pathways can protect neurons from alcohol toxicity. All these findings support the hypothesis that alcohol disturbs Ca^{2+} homeostasis to trigger apoptosis.

The experiments described in Chapter III investigated how the NO signaling pathway through PKG influences intracellular Ca^{2+} release to mediate alcohol resistance. Our studies indicated that activating PKG rescues CGN cultures by blockings the alcohol-induced rapid rise in $[Ca^{2+}]_i$, an effect very similar to that resulting from the IP_3R inhibition observed in Chapter II. Experiments that manipulated temporally the addition of PKG activators in relation to alcohol exposure linked the ability of PKG to prevent the alcohol-induced $[Ca^{2+}]_i$ elevations to its reduction of alcohol toxicity. The marked similarity in neuroprotection provided by PKG activation and by blockade of Ca^{2+} release at the IP_3R supports our hypothesis that NO (via PKG) prevents cell death by attenuating the alcohol-induced disruption of $[Ca^{2+}]_i$. However, BDNF, which does not rely on PKG to provide neuroprotection, failed to block alcohol-induced $[Ca^{2+}]_i$ elevations while preventing alcohol toxicity. This indicates that although PKG blocks alcohol-induced $[Ca^{2+}]_i$ elevations to prevent alcohol toxicity, averting the alcohol-induced $[Ca^{2+}]_i$

elevations is not a necessary requirement of neuroprotection. BDNF may prevent cell death by ameliorating a compromised cellular process (as yet unidentified) downstream from the alcohol-induced disruption of $[Ca^{2+}]_i$.

A Theoretical Model and Future Directions

Figure IV-1 provides a visual representation of how NO signaling may ameliorate neurodevelopmental alcohol toxicity. This figure was generated based on the data presented in chapters II and III, along with previously cited studies regarding alcohol-related Ca^{2+} signaling and NO-mediated neuroprotection mentioned in chapter I. Alcohol by interacting with G proteins stimulates phosphoinositide turnover via PLC to increase IP_3 levels (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2005). Consequently, the elevated IP_3 levels activate IP_3Rs and trigger Ca^{2+} release from internal stores (ER), thus increasing $[Ca^{2+}]_i$ and promoting cell death (Debelak-Kragtorp et al. 2003). NMDA is neuroprotective against neurodevelopmental alcohol toxicity by the NO signaling pathway. Ca^{2+} entry via surface NMDA receptors stimulate nNOS to increase NO levels (Pantazis et al. 1998, Pantazis et al. 1995). In turn, NO activates sGC, subsequently increasing cytosolic cGMP levels and PKG activation (Bonthius et al. 2004, Bonthius et al. 2003, Pantazis et al. 1998). PKG, by phosphorylating IP_3Rs (Koga et al. 1994, Haug et al. 1999), inhibits Ca^{2+} release from internal stores (Fernandez et al. 2005, Morales et al. 2005) and prevents alcohol-induced neuronal death.

Although we have neither demonstrated that alcohol raises intracellular IP_3 levels, nor that PLC inhibition prevents the alcohol-induced Ca^{2+} elevations in our CGN model, these alcohol-related signaling events have already been demonstrated *in vivo* (Debelak-Kragtorp et al. 2003, Garic-Stankovic et al. 2010, Garic-Stankovic et al. 2006, Garic-Stankovic et al. 2005). We have shown that the alcohol-related increase in $[Ca^{2+}]_i$ originates from intracellular stores, depends on the IP_3R , and is a key determinant for alcohol-induced neuronal death. PLC activation is also a necessary requirement of

alcohol-induced cell death. PLC promotes phosphoinositide turnover to elevate IP_3 levels and consequently stimulates Ca^{2+} release from intracellular stores via IP_3 Rs. Since this represent a ubiquitous pathway in mammalian cells (Berridge *et al.* 2003), it may be inferred that alcohol will raise intracellular IP_3 levels, and that PLC inhibition will prevent the alcohol-induced Ca^{2+} elevations in our CGN cultures as well. Nevertheless, a complete analysis of alcohol signaling upstream from the IP_3 R requires demonstrating that the alcohol-induced elevations of IP_3 and Ca^{2+} depend on PLC function in our CGN cell cultures. These experiments would precede future research focused on investigating alcohol's effects on associated G proteins.

An important consideration is also alcohol's effect on RyR activity since RyRs remain another conduit for Ca^{2+} efflux from the ER. As mentioned in Chapter II, blocking IP_3 Rs, but not RyRs, shielded neurons from alcohol toxicity in a preliminary study. To conclude that alcohol-induced neuronal death requires IP_3 Rs rather than RyRs also requires confirming that the RyR antagonist, dantrolene, effectively inhibited RyRs in our experimental preparation. Demonstrating that dantrolene attenuates RyR agonist-induced Ca^{2+} elevations in our CGN cultures would be sufficient. In turn, showing that alcohol-induced Ca^{2+} elevations still occur despite RyR antagonism would support the hypothesis that the alcohol-induced Ca^{2+} surges are IP_3 R-dependent and toxic.

Despite the aforementioned pitfalls, the hypothesis that alcohol induces neuronal death by stimulating a rapid increase in $[Ca^{2+}]_i$ via IP_3 Rs has been supported by the findings presented in Chapter II and is agreement with the theoretical model presented in Figure IV-1. Inhibiting IP_3 Rs or chelating $[Ca^{2+}]_i$ prevents alcohol-induced Ca^{2+} increases and cell death.

Numerous studies (Siler-Marsiglio *et al.* 2005, Ramachandran *et al.* 2003, Vaudry *et al.* 2002, Cheema *et al.* 2000, Pastorino *et al.* 1999, Ramachandran *et al.* 2001, Siler-Marsiglio *et al.* 2006, Wolter *et al.* 1997, Adachi *et al.* 2004, Han *et al.* 2006) have indicated that neurodevelopmental alcohol exposure instigates cell death through

apoptotic mechanisms. The finding that alcohol triggers multiple Ca^{2+} -activated apoptotic pathways that synergistically culminate in cell death offers many exciting avenues of research with the ultimate aim of uncovering the mechanisms responsible for fetal alcohol neurotoxicity. During fetal brain development, neuronal cells acquire a resistance to alcohol toxicity (West et al. 1994). One explanation for this phenomenon is that neurons are purposely susceptible to apoptotic signals that alcohol perturbs; a vulnerability that diminishes during brain development. This susceptibility may be a key component of the neuronal culling process that is essential for normal brain development (Mooney & Henderson 2006). Therefore investigation of these apoptotic pathways may help us understand ultimately why developing neurons during certain vulnerable developmental periods are highly susceptible to alcohol exposure.

Regarding the neuroprotection afforded by the NO signaling pathway, previous work (Bonthius et al. 2004, Bonthius et al. 2003, Bonthius et al. 2006, Bonthius et al. 2002, Pantazis et al. 1995, Pantazis et al. 1998) established that activation of a NO signal transduction cascade protects against alcohol toxicity, reducing alcohol-induced neuronal death *in vivo* and *in vitro*. NO raises cGMP levels (Moncada & Higgs 1995), thus activating PKG (Francis & Corbin 1999). This signaling pathway is also responsible for neuroprotection provided by NMDA against alcohol toxicity, of which nNOS, sGC, and PKG are essential components (Pantazis et al. 1998, Bonthius et al. 2004). We hypothesized that the activated PKG phosphorylates the IP_3R , thereby inhibiting it to prevent Ca^{2+} release. Here we have shown that activation of PKG prevents the alcohol-induced increase in $[\text{Ca}^{2+}]_i$, thereby ameliorating alcohol-induced cell death. Along with the data presented in Chapter III, previous work indicated that stimulation of the NO-signaling can prevent alcohol-induced cell death. Although it can be inferred, demonstrating in our CGN cultures that stimulation of the NO signaling upstream from PKG at nNOS or at sGC can reduce alcohol-induced Ca^{2+} elevations would strengthen

our hypothesis. Indeed, establishing that sGC stimulation attenuates alcohol-induced Ca^{2+} elevations represents a future avenue of research.

In cerebellar tissue, PKG can influence $[\text{Ca}^{2+}]_i$ by phosphorylating the IP_3R (Koga et al. 1994, Haug et al. 1999), which consequently suppresses the IP_3R (Murthy & Makhoulf 1995, Murthy et al. 1993, Murthy & Zhou 2003, Tertyshnikova et al. 1998, Fernandez et al. 2005, Morales et al. 2005). Although the effect of PKG activation on alcohol-induced Ca^{2+} elevations markedly resembles that of IP_3R inhibition, it does not establish that PKG directly neutralizes the IP_3R . Therefore demonstrating a PKG-stimulated increase in IP_3R phosphorylation and a physical interaction between PKG and IP_3Rs in our cell culture model would strengthen this hypothesis. Future phosphorylation and immunoprecipitation studies can establish that PKG physically interacts with IP_3Rs , and that PKG activation elevates IP_3R phosphorylation.

Despite aforementioned pitfalls, the hypothesis that PKG inhibits alcohol-induced $[\text{Ca}^{2+}]_i$ release to prevent cell death has been supported by the findings presented in Chapters II and III and is agreement with the theoretical model presented in Figure IV-1. PKG activation by 8-Br-cGMP ameliorates alcohol-induced Ca^{2+} increases and cell death, whereas PKG inhibition prevents cGMP-mediated neuroprotection.

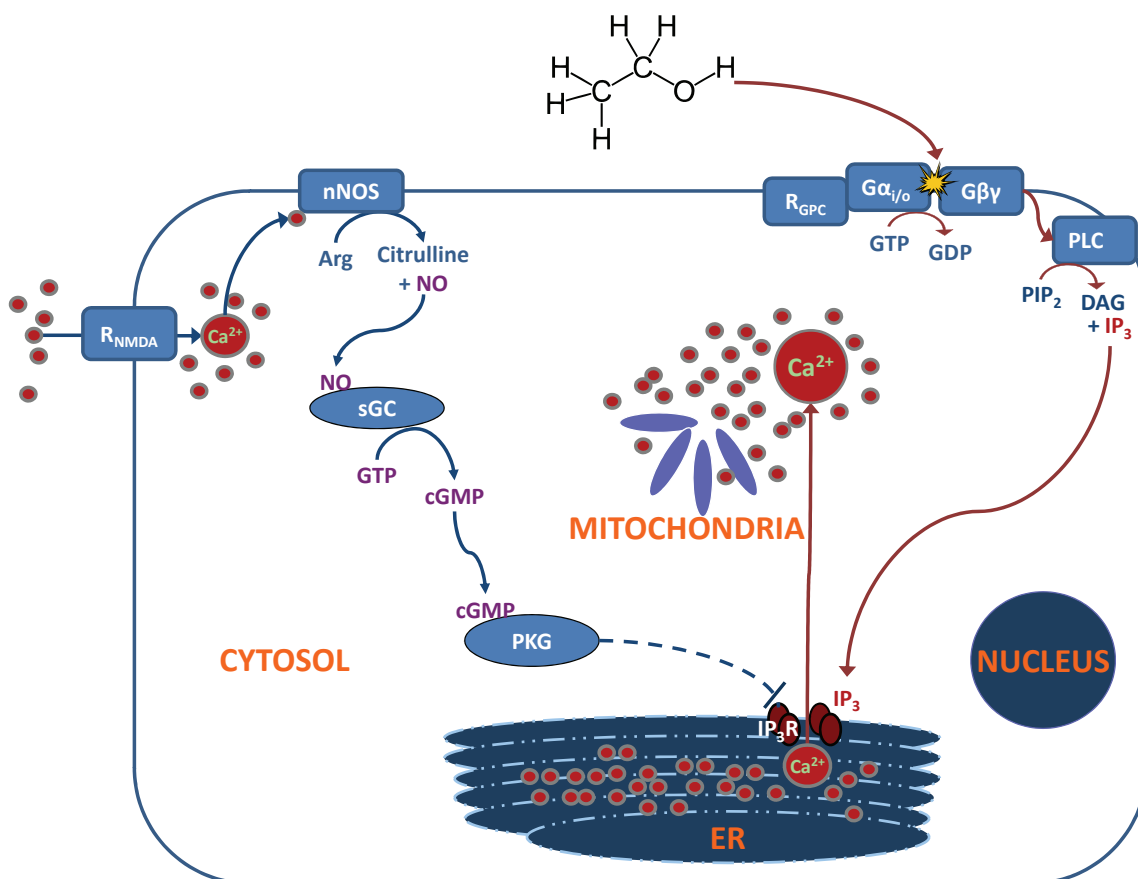


Figure IV-1. A visual representation of our hypotheses. Lines with arrows indicate stimulation, whereas lines with bars indicate inhibition. Dotted lines represent new hypotheses. Displayed are the NO signaling pathway on the left and the PLC-IP₃R pathway to the right of the cell. We hypothesized that PKG inhibits IP₃Rs to prevent Ca²⁺ release from the ER following alcohol's stimulation of PLC.

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