Mechanisms of Ipsapirone-Mediated Neuroprotection of Ethanol-Treated Fetal Rhombencephalic Neurons: Role of Endogenous Antioxidants

Jongho Lee
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MECHANISMS OF IPSAPRONE-MEDIATED NEUROPROTECTION OF ETHANOL-TREATED FETAL RHOMBENCEPHALIC NEURONS:
ROLE OF ENDOGENOUS ANTIOXIDANTS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

JONGHO PETER LEE

CHICAGO, ILLINOIS

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To my dad in heaven
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (Serotonin)</td>
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<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CYP2E1</td>
<td>Cytochrome P-450 2E1</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>EGCG</td>
<td>(-)-epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FAS</td>
<td>Fetal alcohol syndrome</td>
</tr>
<tr>
<td>FASD</td>
<td>Fetal alcohol spectrum disorders</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-trans-nonenal</td>
</tr>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of Kappa B</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LA</td>
<td>Lipoic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MEKK-1</td>
<td>Mitogen-activated protein kinase-1</td>
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<tr>
<td>MEOS</td>
<td>Microsomal ethanol oxidizing system</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor Kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>NF-E2-related factor 2</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidylinositol 3’ kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B/Akt</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>TNF</td>
<td>Tumor necrosis facto</td>
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ABSTRACT

Prenatal exposure to ethanol can cause serious damage to the developing central nervous system. Among the neurotransmitter systems that are affected by early ethanol exposure is the serotonergic system. In fact, both in vivo and in vitro studies demonstrate the vulnerability of the developing serotonergic system to the damaging effects of ethanol. Although ethanol induces apoptotic cell death in these neurons, treatment with a serotonin-1A (5-HT1A) agonist prevents the ethanol-associated apoptosis in developing 5-HT neurons. One mechanism by which 5-HT1A agonists exert their neuroprotective effects appears to involve activation of the phosphatidylinositol 3’kinase (PI-3K) pro-survival pathway and its downstream activation of NF-κB dependent anti-apoptotic genes. Additional NF-κB dependent genes might also be involved with the neuroprotective effects of ipsapirone.

There is considerable evidence that ethanol augments apoptosis by increasing the levels of reactive oxygen species (ROS). In light of the neuroprotective effects of 5-HT1A agonists and their ability to increase expression of NF-κB dependent genes, it was hypothesized that these drugs would also increase the expression of additional NF-κB dependent genes that encode specific antioxidant enzymes. Thus, we investigated the effects of ethanol and the 5-HT1A agonist ipsapirone on ROS levels and on the expression of genes that encode the antioxidant enzymes Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), and catalase. We also investigated whether ipsapirone
treatment augments the activity of these enzymes and whether increased activity of these antioxidant enzymes reduces ethanol-associated apoptosis (Figure 1). These studies used a fluorescent probe to detect changes in ROS, quantitative real-time RT-PCR to measure changes in gene expression, and enzyme assays. In addition, inhibitors of the phosphoinositide-3 kinase (PI-3K) and mitogen-activated protein kinase (MAPK) pathways were used to assess the potential involvement of these pathways with the effects of ipsapirone.

The results of these studies demonstrate that ethanol induces an increase in ROS and that ipsapirone pre-treatment prevents this increase. Time-course studies of the expression and enzyme activity of SOD1, SOD2, and catalase showed that ipsapirone mediates regulation of these enzymes. Ethanol treatment resulted in an early and transient increase in the expression of the three genes. However, expression levels returned to those in unstressed control neurons after prolonged ethanol exposure. Ipsapirone, on the other hand, prevented the latter fall in the expression of the antioxidant enzyme genes. Moreover, addition of EUK-134, which mimics the effects of SOD and catalase, attenuates ethanol-associated apoptosis. Interestingly, inhibitor studies suggested that both the PI-3K → Akt and MAPKK → MAPK pro-survival pathways might be involved with the effects of ethanol and ipsapirone on the expression of the antioxidant genes. In summary, it appears that some of the neuroprotective effects of 5-HT₁A agonists such as ipsapirone are likely to involve the expression of antioxidant enzyme genes.
Alcohol consumption in pregnant women poses a threat to the developing fetus, resulting in life-long disabilities that adversely affect the quality of life of the affected child. Fetal Alcohol Spectrum Disorders (FASD) describes the range of effects that occur in a child as a consequence of maternal alcohol consumption during pregnancy. Fetal Alcohol Syndrome (FAS) presents the most severe form of the disorder. FAS is associated with growth defects, abnormal facial features, and damage to the developing central nervous system, which leads to impairment in cognition, learning, memory, attention, and behavior (Coffin et al., 2005; Riikonen et al., 2005; Streissguth, 1976; Uecker and Nadel, 1998). The Centers for Disease Control and Prevention (CDC) in the United States reported 0.2 to 1.5 cases of FAS for every 1,000 live births. Without a preventative therapeutic treatment, FAS has devastating effects on those affected and their families. It also has a large social impact.

Studies in humans show that maternal consumption of alcohol results in morphological anomalies in cortical and subcortical regions including the brain stem (Bookstein et al., 2001; Clarren et al., 1978; Ferrer and Galofre, 1987; Riikonen et al., 2005). In vitro studies indicate that ethanol neurotoxicity is mediated by increased apoptosis (Druse et al., 2004; Ramachandran et al., 2003). Ethanol augments apoptosis
by increasing the formation of reactive oxygen species (ROS), which leads to oxidative stress. In fact, there is direct evidence showing that ethanol increases ROS (Novack et al., 1982; Thayer and Rubin, 1981) and that accumulation of ROS leads to apoptotic cell death (Druse et al., 2005; Druse et al., 2004; Druse et al., 2006; Li et al., 2001a; Ramachandran et al., 2003). Supplemental antioxidant treatment reduces and prevents the morphological anomalies and prevents apoptosis that is caused by early ethanol exposure (Antonio and Druse, 2008; Chen et al., 2004; Heaton et al., 2004b; Mitchell et al., 1999).

Human and animal studies show that development of the serotonin system is impaired by in utero ethanol exposure (Kim and Druse, 1996b; Rathbun and Druse, 1985; Riikonen et al., 2005; Tajuddin and Druse, 1999; Tajuddin et al., 2003). Serotonin is a classical neurotransmitter, which is produced by serotonergic neurons. Serotonin acts both as a neurotransmitter and as a neurotrophic factor. Because the neurotrophic effects of serotonin are essential to the normal development of the central nervous system (Lauder and Krebs, 1978; Lauder et al., 1985; Mazer et al., 1997; Whitaker-Azmitia et al., 1996), ethanol-associated damage to the developing serotonin system affects serotonin neurons and other CNS neurons (Eriksen et al., 2002; Kim and Druse, 1996a; Sari et al., 2001; Sari and Zhou, 2004; Zhou et al., 2001a; Zhou et al., 2005).

Many of the trophic effects of serotonin are mediated by the serotonin-1A (5-HT$_{1A}$) receptor subtype (Druse et al., 2004; Tajuddin and Druse, 1999). The 5-HT$_{1A}$ receptors are found pre-synaptically on the cell bodies of serotonergic neurons and postsynaptically on target neurons (Siegel et al., 1999). Importantly, 5-HT$_{1A}$ receptor activation exerts protection against ethanol-mediated (Druse et al., 2005; Druse et al., 2006; Kim and
Druse, 1996b; Lee et al., 2009; Tajuddin and Druse, 1999) and hydrogen peroxide-mediated apoptotic cell death (Lee et al., 2005b; Lee et al., 2001). The 5-HT$_{1A}$ agonist ipsapirone is a well-tolerated anxiolytic drug that is used in the United Kingdom and Canada. Ipsapirone treatment appears to prevent ethanol-induced apoptosis in developing 5-HT neurons by activating the phosphatidylinositol 3-kinase (PI-3K) → pAkt pro-survival pathway and its downstream effects, i.e., augmenting the transcription of Nuclear Factor-kappa B (NF-κB) dependent pro-survival genes (e.g. XIAP, Bcl-xl) in vitro (Druse et al., 2005; Druse et al., 2006; Lee et al., 2009). In contrast, a 5-HT$_{1A}$ receptor antagonist inhibits ipsapirone-mediated neuronal protection (Druse et al., 2004; Druse et al., 2006).

In addition to the mentioned pro-survival genes, NF-κB can upregulate other pro-survival genes and genes that encode the endogenous antioxidant enzymes such as Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), and catalase (Pinkus et al., 1996; Rojo et al., 2004; Scandalios, 2005). These antioxidant enzymes maintain redox homeostasis by neutralizing ROS, which are generated during the normal cellular respiratory process. Dysfunction of these enzymes leads to oxidative stress and the development of certain neurological disorders. In contrast, augmenting endogenous antioxidants promotes neuroprotection.

The research described in this dissertation investigated a potential role for the 5-HT$_{1A}$ agonist ipsapirone in detoxifying the highly damaging ROS that are generated after ethanol exposure. Specifically, I investigated the effects of ethanol and ipsapirone on the levels of ROS and on the expression and activity of the antioxidant enzymes catalase, SOD1, and SOD2. The findings provide new information regarding the potential
neuroprotective effects of ipsapirone. These findings may be relevant to the subsequent development of new drugs to prevent brain damage that is caused by prenatal exposure to ethanol.

Hypothesis

Ipsapirone treatment of ethanol-exposed fetal rhombencephalic neurons increases the expression of the genes that encode the antioxidant enzymes Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), and catalase, and the activity of these enzymes; increased activity of these antioxidant enzymes reduces ethanol-associated apoptosis (Figure 1)

Specific Aims

Specific Aim 1

Determine whether ipsapirone prevents accumulation of reactive oxygen species (ROS) in ethanol-exposed fetal rhombencephalic neurons.

Rationale

Although the mechanism(s) by which ethanol exerts its teratogenic effects are not fully understood, several studies show that ethanol can promote the generation and accumulation ROS in multiple cell types (Heaton et al., 2002; Novack et al., 1982; Ramachandran et al., 2003; Thayer and Rubin, 1981). The ethanol-associated increase in oxidative stress promotes apoptosis. That supplemental antioxidants can prevent or reduce ethanol-triggered toxicity supports the important role of ROS in the teratogenic
Figure 1. Schematic diagram of the hypothesis and specific aims that was carried out in this project. (A) Aim 1 determined whether ipsapirone prevents accumulation of reactive oxygen species (ROS) in ethanol-exposed fetal rhombencephalic neurons. (B) Aim 2 demonstrated whether ipsapirone treatment increases the expression of the genes encoding the antioxidant enzymes SOD1, SOD2, and catalase in ethanol-exposed fetal rhombencephalic neurons. A SOD/catalase mimetic EUK-134 was used to determine the effects of the enzymes in preventing ethanol-mediated apoptosis. (C) Aim 3 demonstrated whether the PI-3K pathway is involved with ipsapirone-mediated changes in the expression of NF-κB dependent antioxidant enzymes genes that encode catalase, SOD1, and SOD2 in ethanol-exposed fetal rhombencephalic neurons.
effects of ethanol (Chen et al., 2004; Heaton et al., 2004b; Li et al., 2001a). Previous work from this laboratory also revealed that treatment with free-radical scavenging antioxidants such as EGCG, resveratrol, and lipoic acid reduces the ethanol-associated apoptosis (Antonio and Druse, 2008). There is also evidence that the 5-HT$_{1A}$ agonist ipsapirone can prevent ethanol-associated apoptosis (Druse et al., 2004). In light of the common neuroprotective effects of a 5-HT$_{1A}$ agonist and a classical antioxidant, this aim will determine whether ipsapirone’s neuroprotective effects might involve an attenuation of ethanol-augmented ROS. Thus, this aim will investigate whether ipsapirone can prevent the ethanol-induced generation of ROS.

**Design and Methods**

Because the studies described in this dissertation investigated the effects of ethanol and the 5-HT$_{1A}$ receptor agonist on fetal rhombencephalic neurons, the initial studies established that the 5-HT$_{1A}$ receptor was expressed in this population of neurons. Cultures of fetal rhombencephalic neurons include 5-HT and other developing neurons (Druse et al., 2004). To assess the ethanol-induced generation of ROS two methods, fluorescent imaging and fluorescent spectroscopy, were compared using ROS sensitive dye 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA). In order to determine the ROS-reducing effects of the 5-HT$_{1A}$ agonist ipsapirone, fetal rhombencephalic neurons were treated with ipsapirone either 24 hours prior to the addition of 50 mM ethanol or at the time ethanol was added. The changes in ROS were measured over time by fluorescence intensity using a fluorescent microplate reader.
Specific Aim 2

Determine whether ipsapirone treatment increases the expression of the genes encoding the antioxidant enzymes SOD1, SOD2, and catalase in ethanol-exposed fetal rhombencephalic neurons.

Rationale

Oxidative stress is widely studied as a possible mediator of many neurological disorders [Reviewed in (Hamilton et al., 2004; Sun and Sun, 2001; Sun et al., 2001; Wu and Cederbaum, 2003)]. With respect to alcohol-related disorders, including FASD, ethanol can cause decreased levels of endogenous antioxidants that are involved with the neutralization of ROS. Ethanol can also reduce enzyme activities of three major enzymes SOD1, SOD2, and catalase (Heaton et al., 2003a; Heaton et al., 2003b; Ledig et al., 1981; Reddy et al., 1999). However, increasing levels of endogenous antioxidants moderate oxidative stress, which results in protective outcomes (Jayalakshmi et al., 2005; Li et al., 2001a; Liu et al., 2005). Research shows that overexpression of Sod2 is protective against alcohol-induced cellular damage by attenuating the oxidative stress (Wheeler et al., 2001). In addition, catalase increases cell viability in ethanol treated cells (Gonzalez et al., 2007; Li et al., 2001a). Thus, the changes in the expression of SOD1, SOD2, and catalase may play a role in moderating oxidative stress and contribute to neuroprotection. Interestingly, pharmacological agents such as EUK-134 that mimic SOD/catalase enzyme activity also provide protection (Chen et al., 2004; Rong et al., 1999).

Previous work from this laboratory demonstrated that the 5-HT₁A agonist ipsapirone upregulates expression of genes that encode the pro-survival/anti-apoptotic
proteins XIAP and Bcl-xL (Druse et al., 2005; Druse et al., 2005; Lee et al., 2009). These and several other pro-survival genes are regulated by transcription factor NF-κB. Interestingly, SOD1, SOD2, and catalase are endogenous antioxidant enzymes that neutralize naturally occurring ROS, formed during cellular respiration, and their gene expression is also regulated by NF-κB (Pinkus et al., 1996; Rojo et al., 2004; Scandalios, 2005).

Because ipsapirone provides neuroprotection against ethanol-associated apoptosis and because this drug augments the expression of at least two NF-κB dependent pro-survival genes, ipsapirone might also increase the expression of additional NF-κB dependent genes, i.e., those encoding endogenous antioxidant enzymes. This aim studied the effects of ipsapirone on the regulation of gene expression and enzyme activities of the antioxidant enzymes SOD1, SOD2, and catalase in control and ethanol-exposed fetal rhombencephalic neurons. In addition, the potential neuroprotective effects of pharmacological SOD/catalase mimicry were assessed.

**Design and Methods**

Cultured fetal rhombencephalic neurons were studied in four treatment groups: Control (no ethanol/no ipsapirone), Ipsapirone (no ethanol/ 100 nM ipsapirone), Ethanol (50 mM ethanol/ no ipsapirone), and Ethanol + Ipsapirone (50 mM ethanol/ 100 nM ipsapirone). Since the protective effects of ipsapirone on ethanol-induced apoptosis, i.e. reduction in apoptosis and elevation of pro-survival gene expression, occurred within 24 hours (Druse et al., 2004; Druse et al., 2006), temporal expression profiles of the endogenous antioxidants enzymes were determined to better understand the roles of these
enzymes. Real-time RT-PCR was performed to determine the changes in mRNA after the treatments at various times.

To assess the potential importance of SOD and catalase in ethanol-induced apoptosis, neurons maintained in the presence of 0 or 50 mM ethanol were co-treated for 24 hours with EUK-134, a pharmacological SOD/catalase mimetic. To determine the optimal dose, two concentrations of EUK-134 were used, i.e., 0.5 µM and 5 µM. As previously employed (Antonio and Druse, 2008; Druse et al., 2004), Hoechst 33342 staining was used to detect and to compare apoptotic nuclei (condensed or fragmented) in these treatments.

Specific Aim 3

Determine whether the PI-3K pathway is involved with ipsapirone-mediated changes in the expression of NF-κB dependent antioxidant enzymes genes that encode catalase, SOD1, and SOD2 in ethanol-exposed fetal rhombencephalic neurons.

Rationale

The pro-survival effects that are mediated by 5-HT_{1A} receptors potentially involve several well-known signaling pathways (Abdouh et al., 2004; Adayev et al., 1999; Adayev et al., 2003; Hsiung et al., 2005). One of these pathways involves activation of PI-3K → pAkt and subsequent downstream activation of transcription factor NF-κB (Abdouh et al., 2004; Cowen et al., 1997; Dhandapani et al., 2005). Previous work from this laboratory revealed that the 5-HT_{1A} agonist ipsapirone activates PI-3K → pAkt in cultures of ethanol treated fetal neurons (Druse et al., 2005). Additional studies indicate
that MAPK signaling pathways and crosstalk between other pathways may also be involved with 5-HT\textsubscript{1A} receptor-mediated protection (Adayev et al., 1999; Adayev et al., 2003). Activation of these pro-survival signaling pathways leads to upregulation of genes that encode proteins that promote cell survival. That is, activation of PI-3K $\rightarrow$ pAkt by ipsapirone upregulates the expression of \textit{XIAP} and \textit{Bcl-xL} in ethanol-exposed fetal rhombencephalic neurons (Druse et al., 2006; Lee et al., 2009). Therefore, this aim investigated pathways that might be involved in the activation of the NF-κB dependent antioxidant enzyme genes that encode SOD1, SOD2, and catalase.

\textit{Design and Methods}

To investigate which signaling pathways might be involved with ipsapirone’s effects on fetal rhombencephalic neurons, the PI-3K inhibitor LY294002 and the MAPK inhibitor PD98059 were included in studies in which cells neurons were cultured in the presence of 0 or 50 mM ethanol and/or 0 or 100 nM ipsapirone. As described in Aim 2, real-time RT-PCR was used to assess the changes in mRNA. Appropriate time points were determined on the basis of the results from Aim 2. That is, inhibitor studies were performed at a time when increased gene expression was observed.
CHAPTER 2
REVIEW OF RELATED LITERATURE

Fetal Alcohol Syndrome

In the early 1900s, Europeans reported an association between chronic maternal alcohol consumption and infant mortality (Sullivan, 1900). In 1973, Jones and colleagues (Jones and Smith, 1973) described the teratogenic effects of ethanol in the United States. The extensive abnormalities in children with Fetal Alcohol Syndrome (FAS) included growth deficiency, limb and joint abnormalities, craniofacial abnormalities, and fine motor skill dysfunction (Hanson et al., 1976; Jones and Smith, 1975). Many studies over the subsequent three decades confirmed and extended the original findings. They demonstrated that alcohol ingestion during gestation causes severe abnormalities in FAS offspring (Darby et al., 1981; Naidoo et al., 2006; Smith, 1981; Sowell et al., 2002a). In recent years, there has been considerable effort devoted to further elucidating the effects of ethanol on the developing fetus and investigating the mechanisms that underlie the toxic effects of ethanol.
FAS and FASD nomenclature

In the past, the term FAS was widely used to describe many of the disorders found in children whose mothers ingested alcohol during pregnancy. In a recent effort to provide more clarity, Hoymes et al. (Hoyme et al., 2005) described the spectrum of disorders associated with maternal alcohol consumption as Fetal Alcohol Spectrum Disorders (FASD). The term FASD includes FAS and other alcohol-related developmental disorders.

Diagnosis of FAS and FASD

In early studies of humans, participants were usually screened for FAS based on a history of maternal alcoholism and the presence of additional characteristics, including growth deficiency, certain craniofacial characteristics, and noticeable physical anomalies (small head, limb malformation, etc) (Darby et al., 1981; Hanson et al., 1976; Hayden and Nelson, 1978). Even then, researchers knew that differences in drinking patterns of the mothers (chronic or occasional drinkers, heavy or moderate drinkers) contributed to differences in the severity of alcohol-related deficits in their children [reviewed in (Streissguth et al., 1980)].

In light of well recognized difficulties in identifying FAS and FASD [reviewed in (Sampson et al., 1997)], The Institute of Medicine (IOM) issued a guideline for diagnosing FAS and other alcohol-related effects (IOM-Institute of Medicine (U.S.). Division of Biobehavioral Sciences and Mental Disorders. Committee to Study Fetal Alcohol Syndrome., 1996). The recently revised diagnostic criteria (Figure 2) assigned those affected with FASD to one of six classifications: 1) FAS with confirmed maternal
alcohol exposure, 2) FAS without confirmed maternal alcohol exposure, 3) partial FAS with confirmed maternal alcohol exposure, 4) partial FAS without confirmed maternal alcohol exposure, 5) Alcohol Related Birth Defects (ARBD), and 6) Alcohol Related
Figure 2. A guideline for diagnosing FAS and other alcohol-related effects. The Institute of Medicine (IOM) issued a guideline for diagnosing FAS and other alcohol-related effects. This is an updated version of the guideline published by Hoyme and colleagues (Hoyme et al., 2005)
Neurodevelopmental Disorder (ARND) (Hoyme et al., 2005). For example, FAS with confirmed maternal alcohol exposure presents the most severe diagnosis in which all of the following criteria must be met. This assignment requires confirmed maternal alcohol exposure, evidence of a characteristic pattern of minor facial anomalies (short palpebral fissures, thin vermilion border of the upper lip, smooth philtrum), evidence of prenatal and/or postnatal growth retardation (height or weight less than the 10th percentile, corrected for racial norms, if possible), and evidence of deficient brain growth or abnormal morphogenesis (structural brain abnormalities, head circumference less than 10th percentile).

**Human Studies of FAS**

The Development of Multiple Organ Systems: Effects of *In Utero* Ethanol Exposure

Many of the early studies of humans with FAS focused on specific clinical cases and the description of patients’ congenital anomalies, including growth deficiencies as well as limb and craniofacial malformations (Castells et al., 1981; Hanson et al., 1976; Jones et al., 1974; Jones et al., 1976). These abnormalities are visible indications that are still included in the guidelines of FASD diagnosis (see Diagnosis). In addition to the visible abnormalities in ethanol-exposed children, there is evidence that several developing organs are vulnerable to ethanol toxicity. An early study revealed that cardiac malformations, including interruption in the aortic arch and ventricular septal defects, were found in some infants with FAS (Steeg and Woolf, 1979; Terrapon et al., 1977). There is also evidence of abnormal development of the kidney (Qazi et al., 1979),
skeleton (van Rensburg, 1981), ocular system (Stromland and Sundelin, 1996), and brain
(Clarren et al., 1978) in FAS children. Thus, alcohol consumption during pregnancy has
the potential to adversely affect many aspects of development and to cause lasting
damage that is detected in infancy, childhood, and adulthood. Although only a small
number of children affected by FAS have died, the limited number of postmortem
autopsies confirmed the toxic effects of ethanol on developing brain (Clarren et al., 1978;
Jones and Smith, 1975).

The following sections review the effects of ethanol on the development of CNS.

The Development of the Central Nervous System: Effects In Utero Ethanol Exposure

Prenatal alcohol exposure is particularly harmful to the developing central
nervous system (CNS). In fact, damage to the developing CNS has been a major focus of
FAS research because of the extent of ethanol-associated CNS injury and its long-term
functional consequences. Prenatal alcohol exposure is particularly injurious to the
developing CNS (Hanson et al., 1976; Kennedy, 1984), because it is one of the earliest
organ systems to develop. Also, CNS development is prolonged and the brain is
vulnerable to the damaging effects of ethanol throughout most of gestation.

Neuropathological Studies and Brain Morphology: Effects of In Utero Ethanol Exposure

Early clinical cases and postmortem brain analyses of FAS were reported by
research groups from Europe and the United States (Jones and Smith, 1973; Lemoine et
al., 1968; Sullivan, 1900). Studies from the 1970’s and 1980’s described the brain
dysmorphology that is associated with FAS and FASD. A report by Clarren and
colleagues focused on neuropathological observations from four children and the associated drinking habits of their mothers (Clarren et al., 1978). In two cases, brain malformations positively correlated with the mothers’ self-reported drinking habits: the more alcohol the mother drank, the greater the brain malformations in the child. Additional brain abnormalities were consistent with earlier reports (Hanson et al., 1976; Jones and Smith, 1975). Clarren’s report also showed that the brains of affected children were highly disorganized, due to errors in the migration of neurons and glia (Clarren et al., 1978; Jones and Smith, 1975). Other offspring of chronic alcoholic mothers exhibited dysmorphism of dendritic spines, suggesting dysfunction of the neurons (Ferrer and Galofre, 1987) and microcephaly. In the latter case, severe midline cerebral malformations were present, including a partially fused frontal lobe and the absence of olfactory bulbs and tract, pituitary lobe and anterior corpus callosum (Coulter et al., 1993). Roebuck and colleagues provided a thorough review of brain dysmorphogenesis in FAS children (Roebuck et al., 1998). Taken together there is evidence of substantial and diffuse pathological brain damage in FAS.

Neuropsychological Studies Detect Abnormalities in Fetal Alcohol Offspring

As more pathological cases describing brain dysmorphology were reported, many researchers became interested in the functional consequences of the brain abnormalities. The results of their studies show that there are neurobehavioral changes in children exposed to ethanol in utero (Slavney and Grau, 1978; Streissguth, 1976; Streissguth, 1977; Streissguth et al., 1994; Yellin, 1984). The adverse effects of alcohol on the developing brain are manifested in neuropsychological impairments (Aronson et al.,
1985; Steinhausen et al., 1982; Streissguth, 1976) that include attention deficits (Riikonen et al., 2005), learning difficulties (Coffin et al., 2005), memory impairment (Uecker and Nadel, 1998), and abnormal motor development (Olson et al., 1998). FAS and FASD children showed characteristics of attention deficits that are distinct from those in ADHD children, particularly when tested with an eyeblink conditioning test (Coffin et al., 2005). A deficit in CNS-controlled motor skills and coordination were found in 75% of FASD patients (Connor et al., 2006). Interestingly, some of the neuropsychological deficits are still found in adulthood (Barr et al., 2006; Malisza et al., 2005; Streissguth et al., 1991).

As evidence of cognitive abnormalities, children with FAS have impaired spatial working memory (Uecker and Nadel, 1998), which did not improve with age (Malisza et al., 2005). Also, children with FAS performed poorly on auditory and visual attention tests (Connor et al., 1999), but object memory seemed to be spared (Uecker and Nadel, 1998). Verbal learning deficits, including abnormalities encoding verbal information have been described, too (Mattson et al., 1996). Generally, low IQ scores were found in FASD children, but these abnormalities were not sufficient to completely explain attention and memory deficits (Olson et al., 1998). Irrespective of IQ scores, all FASD children (those having low and normal IQ scores) performed poorly on complex attention, verbal learning, and executive function tests (Kerns et al., 1997).

In addition, to learning deficits and attention abnormalities, many young adults with FASD exhibit psychiatric disorders. Neuro-psychological disorders related to prenatal exposure to alcohol include attention deficit, depression, and anxiety disorders. The latter conclusion was based on the responses of FASD offspring to chronic mild stress and on subsequent changes in stress hormones (Hellemans et al., 2008). In
addition, prenatal exposure to alcohol significantly increases the probability of developing substance abuse/dependence as well as somatoform, paranoid and passive-aggressive traits (Barr et al., 2006).

Neuro-imaging Studies: Brain Dysmorphology and Functional Abnormalities

In several studies, sophisticated neuro-imaging methods were used to provide more detailed information regarding the neuroanatomical changes in FASD children. Undoubtedly, many of these changes are functionally related to neuropsychological conditions in FAS. For example, when FASD children were given response-inhibition tasks and evaluated for attention, the functionally active brain regions in the alcohol-exposed children were different from those in controls. MRI studies showed that the prefrontal cortical regions were more highly activated in FASD children and the right caudate nucleus was less activated (Fryer et al., 2007). MRI studies also showed that the hippocampus, a brain region that is involved with learning and memory, had asymmetrical development in FASD children. That is, the left hemisphere hippocampus (compared to right hemisphere) had a disproportionately smaller volume and received a reduced blood supply (Riikonen et al., 1999). A reduced volume of the basal ganglia was also found (Archibald et al., 2001). In addition, it should be noted that motor function impairment is found in FAS, and 75% of FASD children demonstrated a reduced size of the cerebellum (Bookstein et al., 2006). Moreover, MRI studies consistently found a reduction of brain gray and white matter (Archibald et al., 2001; Gabrielli et al., 1990; Sowell et al., 2002b). One study demonstrated lateral splenial/parietal white matter abnormalities, which could correlate with poor visuomotor integration (Sowell et al.,
While integration of information plays an important role in cognition, abnormalities in the corpus callosum (Bookstein et al., 2001; Bookstein et al., 2002; Bookstein et al., 2002; Riley et al., 1995; Sowell et al., 2001) are among the most prominent findings in studies of FASD children. Imaging techniques facilitated the measurement in the changes of specific neurotransmitter systems. In fact, Riikonen et al. reported that the serotonin system seems to be vulnerable to prenatal exposure to ethanol as shown by a reduction of serotonin content in cortical regions (Riikonen et al., 2005). Additional animal studies, demonstrating ethanol-associated abnormalities in the developing serotonin system are reviewed in a subsequent section.

Critical/Vulnerable Periods of CNS Development: Effects of In Utero Ethanol Exposure

Defining the critical/vulnerable periods for teratogenic effects of alcohol continues to be an important topic in the field fetal alcohol research (Kalmus and Buckenmaier, 1989; Sulik et al., 1981; Yelin et al., 2007). Such studies show that fetal exposure to ethanol negatively affects embryogenesis (Sulik K.K, 2008) as well as fetal development (Smith, 1980). One study showed that maternal administration of ethanol during the preimplantation period causes severe developmental abnormalities in embryos (Padmanabhan and Hameed, 1988). In addition, ethanol administration during gastrulation results in craniofacial dysmorphogenesis (Sulik and Johnston, 1983). Early ethanol exposure also prevents the normal closing of neural tube, which can lead to neural tube defects like dysraphia and anencephaly (Chen et al., 2005). It is not surprising that embryos are particularly susceptible to ethanol because they do not have a functional placenta or liver to properly metabolize ethanol. However, ethanol also exerts
teratogenic effects when it is administered later in gestation. In fact, neuronal cells in different regions of developing brain were vulnerable when ethanol exposure (Druse and Paul, 1988; West, 1993) was confined to the second and third trimesters,

**Animal Models of Fetal Alcohol Syndrome Disorder**

**Importance of Animal Models in the Study of FAS and FASD**

The development of animal models of FAS provides a means by which some of the limitations of human studies can be avoided. For example, the frequent consumption of additional drugs of abuse by mothers of FAS children (Weiner et al., 1983) complicates our understanding of the effects of ethanol on development. However, investigators can readily control the dose, duration of treatment, and other variables in animal studies. Fortunately, observations made using animal models seem to correspond well with human studies [reviewed in (Driscoll et al., 1990)].

Several *in vivo* findings in human and animals studies parallel each other, especially with regard to the neuroanatomical and neurobehavioral effects of ethanol. Both human studies (See Human Studies of FASD) and animal studies demonstrated diffuse effects of alcohol on the developing brain (He et al., 2005; Herrera et al., 2003; Stoltenburg-Didinger and Spohr, 1983; West et al., 2001). Both humans and rats exposed to ethanol early in development demonstrate evidence of a functional impairment of the cerebellum, abnormal cerebellar development (Brown et al., 2008; Coffin et al., 2005; Maier et al., 1999), and reduced cerebellar size (Bookstein et al., 2006) and/or cell number (Maier et al., 1999). In addition, FASD children exhibit impaired eyeblink
behavior, which is indicative of cerebellar abnormalities (Coffin et al., 2005; Jacobson et al., 2008). Rats, exposed to ethanol early in development, also demonstrate impairment of a similar behavior (Brown et al., 2008). Using in vitro studies of the cerebellum, several laboratories proposed possible mechanisms for the ethanol-mediated cerebellar abnormalities (Heaton et al., 2004a; Luo et al., 1997; Ramanathan et al., 1996). It is clear that both in vivo and in vitro animal studies, provide important information regarding possible mechanisms that contribute to CNS damage in FASD.

Additional examples of parallels between human and rodent studies come from investigations of spatial memory and studies of the hippocampus. For example, ethanol-exposed rats require a longer time to learn the Morris water maze task, which assesses spatial short term memory (Clements et al., 2005). Spatial memory abnormalities did not improve with age in humans (Malisza et al., 2005). In the hippocampus, both reduced neurogenesis (Clements et al., 2005; He et al., 2005) and increased apoptosis were detected (Wozniak et al., 2004). Also, human MRI studies showed that the left hemisphere hippocampus had a disproportionately smaller volume and received a reduced blood supply (Riikonen et al., 1999). The noted changes could contribute to functional and behavioral anomalies.

The development of several neurotransmitter systems, including those containing dopamine, GABA, serotonin, acetylcholine, noradrenaline, and glutamate (Druse et al., 1990; Druse et al., 1991; Mooney and Miller, 2001; Riley et al., 2001; Smith, 1979; Tajuddin and Druse, 1999; Tajuddin and Druse, 2001) are adversely affected by in utero ethanol exposure. The effects of ethanol on the development of the serotonin system is of
particular relevance to this dissertation research. Those studies are described in a later section.

**In vivo and In Vitro Animal Studies: Ethanol Promotes Apoptosis**

Several studies show that ethanol augments the apoptosis in developing neurons (Druse et al., 2004; Heaton et al., 2004b; Jacobs and Miller, 2001; Li et al., 2001b; Olney et al., 2002b; Ramachandran et al., 2003). Apoptosis is a programmed cell death that involves receptor-mediated (extrinsic) or mitochondria-mediated (intrinsic) pathways. Both pathways ultimately lead to the fragmentation of DNA, which is facilitated by the executioner caspase-3 [reviewed in (Ryter et al., 2007)]. Ethanol can promote apoptosis by activating either the extrinsic or intrinsic pathway. Along with the visible evidence of apoptotic neurons mentioned above, *in vivo* and *in vitro* treatment with ethanol activates caspase-3 (Jacobs and Miller, 2001; Mooney and Miller, 2001; Olney et al., 2002a) (Mooney and Miller, 2001) and increases the expression of genes encoding the pro-apoptotic members of the Bcl-2 family, e.g, Bax and Bcl-xs (Mooney and Miller, 2001; Moore et al., 1999). Also, *in vitro* ethanol exposure induces DNA laddering, which is indicative of DNA fragmentation and apoptosis (Oberdoerster et al., 1998).

It appears that the ethanol-mediated toxicity might be linked to activation of pro-apoptotic pathways and/or inhibition of pro-survival signaling pathways. For example, ethanol inhibits a neurotrophic effect by blocking the increase in intracellular Ca\(^{2+}\) that is mediated by activation of the N-methly-D-aspartate (NMDA) glutamate receptor subtype (Bhave and Hoffman, 1997). In addition, ethanol inhibits the PI-3K pro-survival pathway that is activated by insulin-like growth factor 1 (IGF-1). This inhibition results
in apoptosis of cerebellar granule neurons (Zhang et al., 1998). Ethanol also promotes apoptosis in primary cultures of developing serotonergic neurons, presumably by reducing the activation of the PI-3K pro-survival pathway (Druse et al., 2005).

*In Vitro* Animal Studies: The mechanisms of Ethanol Toxicity

It can be difficult to use *in vivo* animal models of FASD because it is difficult to get rats to consume high quantities of ethanol. *In vitro* models facilitate use of a controlled treatment; this is useful for assessing specific mechanistic changes, e.g., gene expression, cell signaling, post-translational protein modifications, and DNA damage. As noted previously, potential explanations for the damaging effects of ethanol on the developing hippocampus come from *in vitro* studies done by several researchers. Using *in vitro* models of ethanol exposure, it was suggested that the abnormal cerebellar development might be explained by at least three factors: ethanol’s inhibition of L1-mediated cell adhesion of cerebellar granule cells (Ramanathan et al., 1996); lack of essential neurotrophic factors (Luo et al., 1997); and/or the presence of oxidative stress (Heaton et al., 2004a).

*In vitro* research is also helpful in studies of potential therapeutic agents. For example, *in vitro* studies showed that the protective effects of the 5-HT1A agonist ipsapirone against ethanol-induced apoptosis in developing neurons was mediated in part by stimulation of pro-survival signaling pathways (Druse et al., 2005). In addition, there is *in vitro* and/or *in vivo* evidence from this and other laboratories that antioxidants (Antonio and Druse, 2008; Chen et al., 2004; Heaton et al., 2004b; Siler-Marsiglio et al., 2004) as well as specific neuropeptides (Arevalo et al., 2008; Chen et al., 2005; Parnell et
al., 2007) provide neuroprotection from the damaging effects of ethanol on the developing CNS.

Taken together, both in vivo and in vitro animal studies not only supplement findings in human FASD, but also are useful to elucidate the mechanisms underlying the toxic effects of ethanol and to identify potential neuroprotective agents.

THE SEROTONIN SYSTEM

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine neurotransmitter (Figure 3) that is synthesized from tryptophan. In brain, a large population of serotonergic cell bodies is located in groups in the raphe nuclei. Surprisingly, 5-HT was initially not recognized as a neurotransmitter. A review by Whitaker-Azmitia discusses the history of research on 5-HT. 1) 5-HT was first discovered in chemicals isolated from smooth muscle of the gut. 2) Drs. Twarog and Page first showed the existence of 5-HT in mammalian brain in 1953 (Whitaker-Azmitia, 1999). Since the early discoveries, many studies have shown important behavioral effects of 5-HT. That numerous drugs that act on the 5-HT system are used to treat anxiety, stress and depression emphasizes the involvement of this CNS neurotransmitter with these behaviors.

Development of Serotonin System: 5-HT as a Neurotrophic Factor

Following the discovery that neuronal cell bodies in brain stem produced monoamines (Dahlstroem and Fuxe, 1964), the 5-HT cell bodies (raphe nuclei) and their projections were described in prenatal rat brain (Olson and Seiger, 1972). In the
Figure 3. Chemical structure of serotonin. Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter that contains an indole ring as a backbone structure. It is derived from the precursor amino acid tryptophan.
developing rat CNS, 5-HT neurons are first detected around gestation day 12 (G12), where G=0 corresponds to the day of insemination. (Aitken and Tork, 1988; Lauder and Bloom, 1974; Wallace and Lauder, 1983), and their differentiation peaks between G15 and G16 (Lauder and Krebs, 1978). Ascending projections of 5-HT neurons are detected by G12 (Aitken and Tork, 1988; Lidov and Molliver, 1982a; Wallace and Lauder, 1983) and they continue to develop until the third postnatal week. By G19 the distribution of 5-HT neurons in the fetal rat brain resembles that of the adult (Lidov and Molliver, 1982b; Wallace and Lauder, 1983).

The very early development of the 5-HT system suggested the possibility that 5-HT might also influence CNS development. In support of this neurotrophic role of 5-HT, the Lauder laboratory described several adverse developmental effects that occurred when in vivo 5-HT synthesis was inhibited during critical period of 5-HT development by the tryptophan hydroxylase inhibitor pCPA. This treatment led to a depletion of 5-HT (Lauder et al., 1985) and delayed differentiation of target cells, i.e., those that receive afferent signals from serotonergic neurons (Lauder and Krebs, 1978). 5-HT depletion during synaptogenesis gave rise to decreased synaptic density in the hippocampus, which led to learning deficits (Mazer et al., 1997). A comprehensive review by Whitaker-Azmitia et al. describes the role of 5-HT as an essential neurotrophic factor for normal CNS development and discusses the effects of additional factors on the development of the 5-HT system (Whitaker-Azmitia et al., 1996). In developing rodent brains, the 5-HT$_{1A}$ receptor is also involved with the migration cranial neural crest cells (Moiseiwitsch and Lauder, 1995). Because 5-HT exerts neurotrophic effects on its own and other CNS
neurons, any damage to 5-HT system can have significant adverse effects on the development of CNS.

The Developing Serotonin System: Sensitivity to Ethanol

In addition to the widespread teratogenic effects of ethanol that have been mentioned, studies from this and another laboratory show that ethanol prevents normal development of 5-HT system (Figure 4). Chronic exposure to a 6.6% (v/v) ethanol, through maternal consumption of a liquid diet during gestation damaged the developing 5-HT system. Ethanol-associated abnormalities include: 1) significantly decreased concentration of 5-HT and/or the 5-HT metabolite 5-hydroxyindoleacetic acid in the cortex, cerebellum, and brain stem (Rathbun and Druse, 1985); 2) reduced density of 5-HT reuptake sites and altered density of 5-HT1A receptors (Druse and Paul, 1988; Kim and Druse, 1996b; Tajuddin and Druse, 1988); and 3) fewer 5-HT immunopositive neurons in the raphe region (Tajuddin and Druse, 1999). In vitro studies, such as those described in this dissertation, demonstrated that a 24-hour treatment of ethanol promotes apoptosis in cultures of fetal 5-HT and other rhombencephalic neurons (Druse et al., 2004). Additional studies revealed that the mechanism underlying the adverse effects of early ethanol exposure might involve reduced activity of the PI-3K➔pAkt, pro-survival signaling pathway (Druse et al., 2005; Druse et al., 2004), and the decreased expression of NF-κB pro-survival genes including Bcl-2, Bcl-xl, Xiap, Ciap1, and Ciap2 (Druse et al., 2006). Additional work from another laboratory confirmed the sensitivity of the
developing 5-HT system to the adverse effects of ethanol (Sari et al., 2001; Zhou et al., 2001a).

**Protective Effects of a 5-HT_{1A} Agonist Against Ethanol Toxicity**

Maternal treatment with a 5-HT_{1A} agonist during the period of the peak 5-HT neuronal development, i.e, G13 to G20, prevented the ethanol-associated decrease in 5-HT reuptake sites (Kim and Druse, 1996b) and the altered density of 5-HT_{1A} receptors (Kim et al., 1997) in the terminal regions. Most importantly, when ipsapirone, the 5-HT_{1A} agonist used in this dissertation research, was administered during the same *in utero* period, this treatment prevented the ethanol-associated reduction of 5-HT neurons and of astrocytes in the raphe region of developing rat brain (Tajuddin and Druse, 1999; Tajuddin and Druse, 2001; Tajuddin et al., 2003). Recent *in vitro* studies revealed that ipsapirone could also prevent the ethanol-associated increase in apoptotic 5-HT and other rhombencephalic neurons (Druse et al., 2004). Further *in vitro* studies showed that ipsapirone’s protective effects might involve its ability to prevent the ethanol-associated decrease in PI-3K→pAkt pro-survival signaling (Druse et al., 2005). *In vitro* studies also found that a 24-hour co-treatment with ipsapirone was able to increase expression of NF-κB dependent genes that encode the pro-survival proteins XIAP and Bcl-XL in fetal rhombencephalic neurons (Druse et al., 2006). Activation of additional NF-κB dependent genes that encode pro-survival proteins or antioxidant enzymes could also provide neuroprotection against ethanol. The established and potential neuroprotective effects of ipsapirone could be important clinically, because this anxiolytic drug lacks the
side-effects associated other 5-HT$_{1A}$ agonists currently prescribed (Feighner and Boyer, 1989).

5-HT$_{1A}$ Receptor-Mediated Signaling Pathways

Development of the 5-HT$_{1A}$ receptors begins during gestation in rodents. Expression of the gene encoding the 5-HT$_{1A}$ receptor is detected by G14.5 in several regions of the developing brain and is maintained throughout the development of most of those areas (Bonnin et al., 2006). There is also an in utero peak in the density of 5-HT$_{1A}$ receptors, which occurs at G15-16 (Hillion et al., 1993). This coincides with the peak differentiation period for 5-HT neurons.

Like other members of the 5-HT$_1$ receptor family, the 5-HT$_{1A}$ receptor is negatively coupled to adenylyl cyclase via G$_{i/o}$ proteins. It is also involved in the opening of K$^+$ channels, which leads to neuronal hyperpolarization (Raymond et al., 1999; Siegel et al., 1999). Besides its classic role, the 5-HT$_{1A}$ receptor has been linked to signaling pathways, including those involved with protein kinase C$\alpha$, mitogen activated protein kinase (MAPK), and PI-3K$\rightarrow$pAkt, and NF-$\kappa$B (Abdouh et al., 2004; Adayev et al., 1999; Adayev et al., 2003; Cowen et al., 1997; Hsiung et al., 2005). Importantly, the mentioned signaling pathways are known to promote cell survival. The link of specific pathways to the 5-HT$_{1A}$ receptor appears to be cell-specific.

5-HT$_{1A}$-Mediated Protective Effects

Many studies demonstrated that treatment with 5-HT$_{1A}$ agonists provides
Figure 4. Prenatal ethanol exposure damages developing raphe nuclei. Raphe nuclei contain cell bodies of neurons that produce 5-HT, which exerts neurotrophic effects during the CNS development. Therefore, damage to raphe nuclei, particularly to serotonergic neurons, can lead to abnormal development of the target regions of the developing brain, including the hippocampus, cerebellum, and cortex.
neuroprotection from ischemic damage to neurons (Bielenberg and Burkhardt, 1990; Bode-Greuel et al., 1990; Prehn et al., 1993; Ramos et al., 2004). It also prevented staurosporine-induced apoptosis of hippocampal and cortical cells (Suchanek et al., 1998). In one of the most intriguing studies with regard to this dissertation research, a 5-HT₁A agonist inhibited hydrogen peroxide-induced oxidative stress and neuronal death in developing cortical neurons (Lee et al., 2005b). In addition, protective effects of some other agents seemed to be mediated by the expression of 5-HT₁A receptors (Xu et al., 2007).

Potential Mechanisms of Ethanol Toxicity in Developing CNS

It is clear that alcohol damage to the developing brain and other organs is extensive. Functional signs of brain damage are found in FASD-associated neuropsychological and neurobehavioral disorders. There are intrinsic difficulties associated with studying the mechanisms of FASD in humans, because of differences in clinical, socio-economical, and genetic status of mothers as well as in amount, duration, and timing of alcohol exposure (Goodlett et al., 2005). However, several in vitro and in vivo animal studies provide information regarding the mechanisms that underlie the ethanol-associated damage. One of these mechanisms involves oxidative stress. In addition, the effects of acetaldehyde, a reactive metabolite of ethanol, cannot be ignored.

In a subsequent section, the evidence that ethanol-associated toxicity can be caused by oxidative stress is reviewed. Later sections also review the metabolism of
ethanol, its effects on the mitochondrial electron transport chain (ETC), and generation of oxidative stress that are most relevant to the current dissertation.

**Damaging Effects of Oxidative Stress**

Levels of ROS, produced in normal physiological conditions, are managed by endogenous antioxidants and antioxidant enzymes. The endogenous antioxidants include glutathione (GSH) and several antioxidant enzymes, e.g., catalase, SOD, and the enzymes involved with regeneration of GSH. Unfortunately, the equilibrium between endogenous antioxidants and ROS can be disrupted, causing oxidative stress and cell death. This can happen with the introduction of cellular stressors, e.g., hypoxia, ETC inhibitors, hydrogen peroxide, and ethanol (Abramov et al., 2007; Dabbeni-Sala et al., 2001; Haorah et al., 2005; Jayalakshmi et al., 2005; Mitsumoto et al., 2002; Muresan and Eremia, 1997).

There are two explanations for the accumulation of ROS, which is associated with oxidative stress (Figure 5). First, there can be an upsurge of ROS generation, which is generally induced by toxins, including ethanol. Alternatively, oxidative stress can develop from insufficient neutralization of ROS. A malfunction in the endogenous mechanisms that degrade the naturally generated ROS results in an accumulation of ROS. Elevated levels of ROS can lead to apoptotic cell death (Lee et al., 2005b).

Oxidative stress has been implicated in several disease processes, including neurodegenerative diseases, liver diseases, and heart diseases [Reviewed in (Hamilton et al., 2004; Sun and Sun, 2001; Sun et al., 2001; Wu and Cederbaum, 2003)]. It also appears that at least some of the adverse effects of ethanol on developing brain involve
oxidative stress. Oxidative stress could arise from uncoordinated neutralization of ROS (Del Maestro and McDonald, 1987) or from a low level of endogenous antioxidants (Mavelli et al., 1982), thus leaving the developing brain vulnerable to oxidative stress.

A genetic form of the human disease Amyotrophic Lateral Sclerosis (ALS) illustrates the latter explanation. That is, a genetic mutation in the gene encoding Cu/Zn-SOD, one of the endogenous antioxidant enzymes, appears to be associated with oxidative stress and the ALS pathology (Rosen, 1993). In summary, augmented generation and/or inadequate degradation of ROS can lead to accumulation of ROS that will ultimately result in oxidative stress. It is important to recognize that in many situations the two scenarios interact.

There are numerous examples of ethanol-associated ROS in brain cells and of a link between the increased ROS and cellular damage and death. Ethanol can both promote the formation of ROS (Li et al., 2001a) and reduce the levels of endogenous antioxidants such as GSH (Maffi et al., 2008; Watts et al., 2005). ROS accumulation/oxidative stress can ultimately result in cell death [reviewed in (Ryter et al., 2007)]. In fact, the Henderson laboratory demonstrated that production of ROS precedes the ethanol-mediated induction of apoptosis (Ramachandran et al., 2003). That is, ethanol treatment rapidly increased generation of ROS in cultured cortical neurons and continued to raise ROS levels (Ramachandran et al., 2003). These changes resulted in apoptotic cell death. In addition, in vitro treatment of neural crest cells with ethanol increases production of several ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals. The increased ROS resulted in loss of cell viability (Chen and Sulik, 1996; Davis et al., 1990).
Figure 5. Redox equilibrium and oxidative stress. In general, normal cells can maintain redox equilibrium by balancing the production of ROS and the expression of antioxidant enzymes, AnOx (Top). However, due to the introduction of a stressor such as ethanol, an upsurge of ROS production and accumulation can shift the balance resulting in oxidative stress (Middle). Alternatively, a stressor can cause insufficient expression of antioxidant enzyme to shift the balance toward oxidative stress.
That ethanol can induce ROS generation in multiple areas of the developing brain, including cerebellum (Heaton et al., 2002) and cortex (Haorah et al., 2008; Ramachandran et al., 2003), shows that an ethanol-associated increase in ROS adversely affects multiple brain areas.

As evidence of the ethanol-associated ROS, there are increased levels of the lipid peroxidation product 4-hydroxynonenal (HNE) in cultured rat (Ramachandran et al., 2001) and human neurons (Haorah et al., 2008). The association between an ethanol-mediated increase in cell death and an increase in other lipid peroxidation products has been reported (Sun et al., 1997; Uysal et al., 1989).

The ability of antioxidants to reduce ethanol-mediated toxicity provides indirect evidence that oxidative stress plays a role in the adverse effects of ethanol. For example, ascorbic acid treatment inhibited ethanol-induced ROS production and prevented growth retardation and microencephaly in ethanol exposed embryos (Peng et al., 2005). Also, the ethanol-associated increase in ROS generation and loss of cell viability were prevented by catalase in PC12 cells (Sun et al., 1997). Furthermore, studies from this laboratory demonstrated that ethanol-associated apoptosis of fetal rhombencephalic neurons was prevented by co-treatment with several different types of antioxidants (Antonio and Druse, 2008).

Ethanol can particularly intensify the generation of ROS because 1) metabolism of ethanol by CYP2E1 produces mitochondria-independent ROS (reviewed later), 2) ethanol inhibits the mitochondrial electron transport chain, causing a leak of electrons which promotes the formation of ROS (reviewed later), and 3) “1” and “2” further
damage mitochondria, which results in more ROS production. Importantly, Lee et al. showed that hydrogen peroxide promoted generation of ROS, decrease in mitochondrial integrity, and activation of apoptosis, all of which were potentiated by ethanol (Lee et al., 2005a).

The specific mechanisms by which ethanol can increase oxidative stress are discussed in subsequent sections.

**Metabolism of Ethanol**

Hepatic oxidation of ethanol by alcohol dehydrogenase (ADH) is the primary pathway by which ethanol is metabolized. In this pathway, the cytosolic enzyme ADH catalyzes the conversion of ethanol to acetaldehyde. Then aldehyde dehydrogenase (ALDH) converts acetaldehyde to acetate (Figure 6). However, there are additional enzymes that metabolize ethanol. A review of the microsomal ethanol-oxidizing system (MEOS) described inducible ethanol metabolism by the microsomal enzyme cytochrome P-450 2E1 (CYP2E1) (Lieber, 1999).

Although the liver is the major site of ethanol metabolism, the brain possesses several pathways to metabolize ethanol. Both ADH (Giri et al., 1989; Kerr et al., 1989; Mandel et al., 1980; Martinez et al., 2001), and CYP2E1 are present in brain (Warner et al., 1988; Warner and Gustafsson, 1994; Wickramasinghe, 1987). In addition, catalase, a peroxisomal enzyme normally involved in conversion of hydrogen peroxide to water, can metabolize ethanol in brain (Cohen et al., 1980; Zimatkin and Lindros, 1996). A comparative analysis of the ethanol-metabolizing enzymes ADH, CYP2E1, and catalase
revealed that catalase is the primary enzyme involved with ethanol metabolism in rat brain (Zimatkin et al., 2006). That is, when ethanol was perfused into the lateral ventricle of living rats, acetaldehyde was formed. However, brain metabolism of ethanol was inhibited by a catalase inhibitor. In fetal brain, it is possible that additional unidentified enzymatic pathways might metabolize ethanol. This possibility was suggested by studies in which there was a rapid accumulation of acetaldehyde even in the absence of co-factors that are required by ADH, CYP2E1, and catalase (Person et al., 2000).

All the enzymes mentioned in the previous paragraph catalyze ethanol metabolism and generate acetaldehyde (Crabb and Liangpunsakul, 2007), which has been proven to be toxic to the nerve cells. The fetus is exposed to acetaldehyde, because maternal acetaldehyde diffuses into the fetus through the placenta (Karl et al., 1988). Even preimplantation embryos, i.e., before the formation of a placenta, are vulnerable to the toxic effects of acetaldehyde (Checiu et al., 1984). Acetaldehyde induced cell death in cultured neurons from the developing rat midbrain (Lee et al., 2005) and caused abnormal embryonic growth and development (Giavini et al., 1992). Most of the acetaldehyde-mediated toxicity is derived from the ability of this reactive molecule to form adducts with macromolecules, i.e., DNA, protein, and lipids. Acetaldehyde can bind to DNA and cause genotoxicity (Wang et al., 2000). In fact, both acute and chronic ethanol treatment in vivo caused DNA damage in several brain regions, including the cerebellum and hippocampus (Guo et al., 2007). In addition, the acetaldehyde adducts of
Figure 6. Metabolism of ethanol. Metabolism of ethanol is well characterized in liver. Alcohol dehydrogenase (ADH) converts ethanol into acetaldehyde, which is associated with ethanol-mediated toxicity. Acetaldehyde dehydrogenase (ALDH) converts acetaldehyde into acetate. CYP2E1 is the microsomal ethanol oxidizing system (MEOS) associated enzyme that is induced after chronic alcohol consumption. Catalase can also oxidize ethanol. Interestingly, catalase appears to be the main enzyme that metabolizes ethanol in brain {1304 Zimatkin, S.M. 2006;}. 
proteins and lipids are toxic to cells (Nakamura et al., 2003; Niemela et al., 1998; Niemela et al., 2002). Taken together, metabolism of ethanol, which occurs in the brain, produces acetaldehyde. Acetaldehyde production, in part, could contribute to ethanol-mediated cellular toxicity.

**Reactive Oxygen Species and Endogenous Antioxidants**

**Reactive Oxygen Species**

Reactive oxygen species are oxygen-containing molecules that are highly reactive radicals with an unpaired electron on the oxygen molecule or non-radicals with a highly reactive state. Examples of ROS include the superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide, and peroxynitrite. Normal cellular respiration generates ROS such as the superoxide anion, which is a precursor to generation of additional reactive species [reviewed in (Temple et al., 2005)]. In order to combat increases in cellular ROS, cells have endogenous detoxification systems in which normally generated ROS are converted to a non-reactive end product, i.e., water. This process involves enzymes such as SOD1, SOD2, catalase, and glutathione peroxidase [reviewed in (Temple et al., 2005)] (Figure 7).

**Superoxide and Superoxide Dismutase.** During mitochondrial ETC-mediated energy production in normal physiological conditions, electrons can leak into the matrix or out of the mitochondria (Curtin et al., 2002; de la Monte et al., 2001; Fleury et al.,
2002; Mansouri et al., 2001) (Figure 8). Leaked electrons can be directly transferred to molecular oxygen, generating superoxide anion (O$_2^-$). Although not the most reactive ROS, superoxide has the potential to generate other highly reactive species. One such species is the hydroxyl radical (\'OH), which is generally formed from hydrogen peroxide. Superoxide is a ROS itself and an important precursor to other reactive species. SOD is a metal-containing enzyme that catalyzes the conversion of two superoxide ions into hydrogen peroxide (H$_2$O$_2$). There are three isoforms of SOD. The copper/zinc containing form of SOD (Cu/Zn-SOD or SOD1) is located in the cytosol, while the manganese containing SOD (Mn-SOD or SOD2) form is found in mitochondria. SOD3, another copper/zinc SOD, is located in extracellular space.

**Hydroxyl Radical.** As previously mentioned, the hydroxyl radical is an extremely powerful oxidant with high oxidizing potential (Temple et al., 2005) that can react immediately with nearby molecules. For example, polyunsaturated fatty acids react with the hydroxyl radical to produce the toxic byproduct 4-hydroxynonenal (4-HNE) (Girotti, 1998). Other studies suggested that 4-HNE also increased neuronal vulnerability to oxidative stress and induced apoptosis in neurons (Keller et al., 1999; Kruman et al., 1997; Malecki et al., 2000).

**Hydrogen Peroxide, Catalase, and Glutathione Peroxidase.** As noted, hydrogen peroxide (H$_2$O$_2$) is generated as a consequence of superoxide metabolism by SOD. SOD
Figure 7. Degradation of ROS by endogenous antioxidant enzymes. Cells are equipped with endogenous antioxidants to prevent accumulation of ROS. The superoxide anion is reduced by superoxide dismutase to hydrogen peroxide. Hydrogen peroxide is further reduced by either catalase or glutathione peroxidase, producing water as a final product. Figure 7 (A) shows the overall reactions in a flow chart, while Figure 7 (B) shows individual reactions carried out by each enzyme.
Figure 8. Mitochondrial electron transport chain (ETC) related ROS generation and neutralization. Mitochondrial ETC is one of the sites of ROS production, even in normal cells. Electron leakage can occur and superoxide anion (O$_2^-$) can be formed in the mitochondrial matrix or cytosol. In general, ETC complexes I and III are believed to be the sites of electron leakage. There are endogenous antioxidant enzymes that help degrade toxic ROS from accumulating. Normally, SOD2 and glutathione peroxidase (GPO) are found in the mitochondrial matrix while SOD1 and catalase are located in cytosol.
catalyzes the formation of hydrogen peroxide from two superoxide radicals. Due to its relative stability (Table 1), hydrogen peroxide has been used as an exogenous agent to induce oxidative stress, i.e. accumulation of ROS, (Jakubowski and Bartosz, 2000; Oyama et al., 1994). However, hydrogen peroxide can also be metabolized through the Fenton or Haber-Weiss reactions to form the hydroxyl radical (Figure 9) or it can be reduced to the non-reactive water molecule by the actions of catalase and/or glutathione peroxidase. Catalase, which is mainly found in peroxisomes, is said to be “one of the most active catalysts produced by nature”; it has a high affinity for hydrogen peroxide and can rapidly detoxify this molecule (Scandalios, 2005).

**Possible sources of reactive oxygen species: control conditions versus ethanol treatment**

As noted, cells normally generate ROS as part of the mitochondrial process of energy production [reviewed in (Temple et al., 2005)]. In the following section two major sources of superoxide that can trigger the production of additional ROS will be discussed.

**Mitochondrial electron transport chain (ETC).** In aerobic cells, oxidative phosphorylation accounts for 90-95% of energy production; this process involves mitochondrial utilization of oxygen. Located in the inner membrane of the mitochondrion, the ETC generates a proton gradient that drives the production of ATP (Figure 8). The ETC is comprised of four oxidoreductase complexes (complex I, II, III, IV). Complex I oxidizes NADH and transfers protons to inner membrane space. This
Table 1. Reduction Potential of Major Reactive Oxygen Species. The reduction potential of ROS indicates its ability to oxidize a nearby molecule. The hydroxyl radical is more highly reactive than its precursor hydrogen peroxide. In contrast, superoxide is not as reactive as its byproduct hydrogen peroxide. Therefore, superoxide is a substrate, which is a precursor to more highly reactive ROS.

<table>
<thead>
<tr>
<th>Reactive Oxygen Species</th>
<th>Reduction Potential (E°/mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl radical</td>
<td>+2310</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>+320</td>
</tr>
<tr>
<td>Iron complex</td>
<td>+120</td>
</tr>
<tr>
<td>Superoxide</td>
<td>-330</td>
</tr>
</tbody>
</table>
Figure 9. Production of the hydroxyl radical. Haber-Weiss and Fenton reactions potentiate the generation of the hydroxyl radical from superoxide and hydrogen peroxide.

\[
\text{H}_2\text{O}_2 + \text{O}_2^- \overset{\text{Fe}^{3+}}{\longrightarrow} \text{O}_2 + \text{OH}^- + \text{OH}^-
\]

\text{<Haber-Weiss Reaction>}

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \overset{\text{Fe}^{3+}}{\longrightarrow} \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

\text{<Fenton Reaction>}

ETC process also generates two electrons, which are transferred to coenzyme Q (also known as ubiquinone) through a semiquinone free radical intermediate. Ubiquinone then carries the electrons to complex III, where these electrons are transferred to cytochrome C. Cytochrome C carries the electrons to complex IV, which transfers them to molecular oxygen, generating water in the matrix.

It is believed that mitochondria can generate ROS, due to insufficient transfer of electrons and electron leakage into the matrix. Within the matrix the electrons will react with oxygen to produce the superoxide anion, which can produce additional ROS (Curtin et al., 2002; de la Monte et al., 2001; Fleury et al., 2002; Mansouri et al., 2001). Complex I and complex III appear to be the major sites of ROS formation. As mentioned, semiquinone, produced by electron transfer in complex I, is a free radical intermediate, and thus has the ability to generate the superoxide anion (Boveris et al., 1976). High production of mitochondrial hydrogen peroxide was reported during oxygen consumption, and this process was associated with complex I (Liu et al., 2002). However, the ROS increase was prevented by pre-treatment with coenzyme Q (Somayajulu et al., 2005).

A comprehensive review by Cahill et al. discussed the effects of ethanol on mitochondria dysfunction (Cahill et al., 2002). Growing evidence suggests that ethanol potentiates mitochondrial ROS generation by negatively affecting the ETC. Chronic ethanol treatment leads to decreased ETC respiratory rates (Novack et al., 1982; Thayer and Rubin, 1981). In fact, ethanol inhibited complex I-, II-, and III-dependent oxidative processes. The latter effects were reversible (Kayser et al., 2003). Studies that used
inhibitors of ETC complexes also demonstrated ethanol-associated ROS production, particular involving complexes I and III (Bailey et al., 1999). Interestingly, ethanol further affects the health of mitochondria by damaging mitochondrial DNA, mitochondrial ribosomes, and mitochondrial membrane permeability (de la Monte et al., 2001; Holmuhamedov and Lemasters, 2009; Pastorino et al., 1999; Patel and Cunningham, 2002; Yan et al., 2007).

**Microsomal CYP2E1.** The normal physiological function and the pathological function of CYP2E1 are discussed in depth by Cederbaum (Cederbaum, 2006). CYP2E1 appears to be involved with the development of alcoholic liver disease. As mentioned in a previous section (See Ethanol Metabolism), the microsomal ethanol-oxidizing system (MEOS) can metabolize ethanol by an enzyme called CYP2E1. While inhibition of CYP2E1 prevented alcoholic liver disease (Sun et al., 2001), overexpression of CYP2E1 resulted in a significantly increased level of hydrogen peroxide (40%-50% increase) (Mari and Cederbaum, 2000). According to Cederbaum, production of the hydroxyl radical occurs in microsomes, and oxidation of ethanol potentiates its production (Cederbaum, 2006). Even though a great deal of research focused on hepatic CYP2E1’s contribution to oxidative stress in liver, there is also evidence that CYP2E1 is expressed in several brain regions, including the cortex, cerebellum, dentate gyrus, and hippocampus (Upadhya et al., 2000). Importantly, ethanol significantly increased CYP2E1 expression in all these brain areas as well as in astrocytes (Montoliu et al.,
Therefore, it is likely that CYP2E1 plays a significant role in mediating oxidative stress and augmenting cellular toxicity.

**Increased ROS Can Potentially Influence Cell Signaling**

Even though most of the toxic effects of ROS have been attributed to oxidative stress and cell death, there is evidence that ROS participate in cell signaling (Hancock et al., 2001). ROS-mediated cell signaling resembles that of other second messengers. ROS are small molecules. They are quickly metabolized, and they are mobile within the cell. A comprehensive review discussed multiple sites at which ROS could influence cell signaling (Figure 10). These sites include transcription factors, growth factor signaling, receptor mediated phosphorylation, and lipid-mediated signaling [reviewed in (Poli et al., 2004)]. One of the more interesting effects of ROS is on transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and NF-E2 related factor (NRF-2), because they have been associated with the transcription of several antioxidant genes (Dong et al., 2008; Pinkus et al., 1996; Rojo et al., 2004). For instance, acute exercise-associated ROS activated the translocation and binding of NF-κB subunits to DNA (Ji et al., 2004). This resulted in the upregulation of endogenous antioxidants (Hollander et al., 2001). Addition of hydrogen peroxide also activated NF-κB through the canonical pathway (phosphorylation-mediated degradation of IκB that otherwise sequesters NF-κB) (Schreck et al., 1991), and through an IκB-independent mechanism. The latter mechanism involved the modulation of DNA-binding subunits (Poli et al., 2004). In fact, NF-κB activation showed sensitivity to redox changes that did not depend on
Figure 10. ROS activation of transcription factors can lead to gene expression. ROS is a small and mobile molecule that has been studied as a possible second messenger to activate signaling pathways. This activation can lead to subsequent activation of transcription factors. Also, some of these transcription factors are likely to be sensitive to redox status and can be directly activated by ROS.
IκB degradation (Canty et al., 1999; Glineur et al., 2000). Others also demonstrated that ROS could activate additional signaling pathways upstream of NF-κB pathway, including those involving mitogen-activated protein kinase (MAPK) (Kanterewicz et al., 1998), c-Jun NH₂-terminal kinase (JNK) (Crossthwaite et al., 2002), and PI-3K→pAkt (Butts et al., 2003). This topic is relevant to this dissertation, which studied the effects of ethanol and ipsapirone on the expression of three NF-κB dependent genes that encode antioxidant enzymes.
CHAPTER 3

IPSAPIRONE ASSOCIATED REDUCTION OF REACTIVE OXYGEN SPECIES IN ETHANOL-EXPOSED FETAL RHOMBENCEPHALIC NEURONS

Abstract

Recent studies indicate that apoptosis significantly contributes to ethanol-associated neuronal cell death in liver, lung, and brain (Brown et al., 2001; Ikegami et al., 2003; Szuster-Ciesielska et al., 2009). One factor that triggers apoptosis is the increased levels of reactive oxygen species (ROS) that can accompany alcohol exposure and other stresses. Previous findings demonstrated that serotonin-1A (5-HT₁A) agonists prevented apoptosis in the developing neurons after ethanol exposure (Tajuddin and Druse, 2001). One potential mechanism by which a 5-HT₁A agonist such as ipsapirone exerts its neuroprotective effects is through an up-regulation of the expression of the NF-κB dependent pro-survival genes such as Xiap and Bcl-XL (Druse et al., 2006). The present study shows that an acute in vitro treatment with ethanol rapidly increased the generation of ROS, measured using an intracellular ROS-sensitive fluorescent dye, in fetal rhombencephalic neurons. The acute ethanol-associated increase in ROS was not prevented by ipsapirone co-treatment of these neurons. However, the increased ROS was prevented in neurons that were pre-treated for 24 hours with ipsapirone before the addition of ethanol. Thus, 5-HT₁A agonist-associated pro-survival effects may also involve reduction of ethanol-induced ROS and oxidative stress.
Introduction

Even though ethanol-induced apoptotic cell death in the developing central nervous system is well documented, the underlying mechanism has not been fully elucidated. Considerable evidence suggests that one possible mechanism of ethanol-associated apoptotic death of neurons is mediated by oxidative stress (Heaton et al., 2003c; Perez et al., 2006),

Ethanol can augment oxidative stress by promoting the formation of ROS (Li et al., 2001a) and by reducing the levels of endogenous antioxidants such as GSH (Maffi et al., 2008; Watts et al., 2005). Evidence of ethanol-associated oxidative stress have been found in vivo and in vitro, and in tissue from multiple brain areas (Haorah et al., 2008; Heaton et al., 2002; Ramachandran et al., 2003). As shown by the Henderson laboratory, an elevation in ROS precedes the ethanol-mediated induction of apoptosis (Ramachandran et al., 2003). Other laboratories also showed that increased ROS resulted in a loss of cell viability (Chen and Sulik, 1996; Davis et al., 1990). Additional evidence that oxidative stress plays a role in the adverse effects of ethanol comes from studies that showed that antioxidants and/or antioxidant enzymes can reduce ethanol-mediated toxicity (Antonio and Druse, 2008; Peng et al., 2005; Sun et al., 1997). Taken together, ethanol treatment increases ROS, and this increase can precede ethanol-mediated apoptosis in developing neurons.

Oxidative stress occurs when high levels of ROS that are not sufficiently dealt with and thus accumulate. In general, there are two ways that ROS can accumulate. ROS levels can rise if there is decreased detoxification of ROS due to lower levels of antioxidant enzymes (Naziroglu et al., 2003). This possibility will be examined in the
next chapter. Alternatively, ROS generation could be augmented following treatment with a stressor such as ethanol (Augustyniak and Skrzydlewska, 2009). The latter possibility is examined in this chapter.

In both in vivo and in vitro studies this laboratory showed that 5-HT\textsubscript{1A} agonists exert neuroprotection against ethanol-induced loss of neurons (Druse et al., 2005; Druse et al., 2004; Druse et al., 2006; Tajuddin and Druse, 1999; Tajuddin and Druse, 2001). In fact, an in vitro study showed that co-treatment of fetal rhombencephalic neurons with the 5-HT\textsubscript{1A} agonist ipsapirone prevented ethanol-induced apoptosis and that a receptor antagonist blocked the neuroprotective effects of ipsapirone (Druse et al., 2004). Interestingly, a study from another laboratory suggested that one mechanism underlying the protective effects of a 5-HT\textsubscript{1A} agonist involved its ability to attenuate oxidative stress that was caused by hydrogen peroxide, a major ROS within a cell (Lee et al., 2005b). Because 5-HT\textsubscript{1A} agonists can prevent apoptosis (Ahlemeyer and Krieglstein, 1997; Suchanek et al., 1998), including ethanol-induced apoptosis (Druse et al., 2004), it is possible that the anti-apoptotic effects of 5-HT\textsubscript{1A} agonists might involve reducing the ethanol-associated ROS increase.

The current study measured intracellular levels of ROS in control and ethanol-treated fetal rhombencephalic neurons. This study also evaluated the potential of the 5-HT\textsubscript{1A} agonist ipsapirone to prevent the ethanol-associated rise in ROS. In order to measure intracellular ROS in the culture system used by this laboratory, a modified method was developed that utilized the ROS sensitive fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCF-DA) (Wang and Joseph, 1999).
Materials and Methods

Fetal Rhombencephalic Neuronal Cultures

The brain region which contains the developing 5-HT neurons (Konig et al., 1988), i.e., the fetal rhombencephalon, was removed (Eriksen et al., 2002) from timed-pregnancy Sprague-Dawley rats (175-185 grams) at gestation day 14 (G14), where G=0 corresponds to the day of insemination. Throughout the dissection procedure, tissue samples were kept ice-cold in Hank’s balanced buffered solution (HBSS), and sterile instruments and techniques were used. As previously described by this laboratory (Druse et al., 2005; Druse et al., 2004; Druse et al., 2006; Druse et al., 2007), rhombencephalic tissue was mechanically disaggregated before seeding on the 1st day in vitro (DIV 1). Cells were seeded onto either poly-D-lysine coated cover glass (Corning, Corning, New York) for confocal microscopy, chambered slides (Nunc, Rochester, NY) for immunohistochemistry and fluorescence microscopy, or 96-well plates (Corning, Corning, New York) for the fluorescent microplate reader. The seeding density varied based on the size of the culture plate: 8x10^6 cells/plate (55 cm^2), 3x10^5 cells/chamber slide (1.8 cm^2), or 4x10^4 cells/well (0.32 cm^2). Cultures were maintained in a neuron-specific chemically modified media (CMM) that included Dulbecco’s Minimal Essential Media/F12 (DMEM/F12) media (Invitrogen, Carlsbad, CA), hydrocortisone-21 sulfate (Sigma, St. Louis, MO), Basal Medium Eagle Vitamin Solution (Sigma, St. Louis, MO), antibacterial agent gentamicin sulfate (Honegger and Monnet-Tschudi, 1997), B27 serum-free medium supplement (Invitrogen, Carlsbad, CA) (Brewer et al., 1993), and 0.25% fetal bovine serum (FBS) (Druse et al., 2004). After 24 hours, 0.4 µM cytosine...
arabinoside (Sigma, St. Louis, MO) was added to arrest gliogenesis. Cells were grown in control media (no ethanol) for 5 days, with media changes made on alternate days.

**Immunohistochemistry**

Fetal rhombencephalic neurons were seeded on DIV 1 as described above. On DIV 5 cultures were washed with warm PBS and fixed with 4% paraformaldehyde. The antibody for the 5-HT<sub>1A</sub> receptor (ImmuoStar, Hudson, WI) was diluted 1:200 in PBS. A Vectastain secondary antibody kit (Vector Labs, Burlingame, CA) and DAB substrate kit (Vector Labs, Burlingame, CA) were used to visualize the immunostained receptors.

To assess the contribution of astrocytes to the cultures immunohistochemistry was performed using an antibody to glial fibrillar acidic protein (GFAP). This method confirmed that astrocytes comprised < 5% of the cells in these neuronally-enriched cultures. All animal care and use procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Loyola University Chicago, Stritch School of Medicine.

**Measurement of Reactive Oxygen Species Generation**

*Fluorescent Microplate Reader and Confocal Microscopy.* Fetal rhombencephalic neurons were seeded on DIV 1 as described above. On DIV 5 or DIV 6 cultures were washed with warm PBS with Ca<sup>2+</sup>/Mg<sup>2+</sup> (Thermo Scientific, Waltham, MA), and membrane permeable 50 µM H<sub>2</sub>DCF-DA (Sigma, St. Louis, MO) was added. Cells were placed in an incubator at 37 °C for 30 minutes. During this period H<sub>2</sub>DCF-DA becomes hydrolyzed and impermeable. Cells were washed once or twice with PBS
before the addition of the treatments. Treatments included 0, 10, 25, or 50 mM ethanol, 0 or 200 µM hydrogen peroxide, or 0 or 100 nM ipsapirone. As a reference, 50 mM ethanol is equivalent to 230 mg/dL, which is a commonly seen blood alcohol level in binge drinkers. For studies using the fluorescent microplate reader (Molecular Devices, Sunnyvale, CA), readings were taken immediately after the addition of treatment. For confocal imaging, the cover glass was placed on a special chassis that had a solution chamber for live cell imaging. Live cell images were taken using a Zeiss LSM-510 confocal microscope (Zeiss, Thornwood, NY).

Fluorescence microscopy was used to determine the effects of a 24-hour pre-treatment with ipsapirone on ROS levels in cultures subsequently treated with 0 or 50 mM ethanol for 2 hours. In these studies, the Pre-Ipsapirone treatment group was treated on DIV5 with 100 nM ipsapirone for 24 hours. This concentration of ipsapirone promotes the development of 5-HT neurons (Whitaker-Azmitia et al., 1996) and prevents apoptosis in fetal rhombencephalic neurons (Druse et al., 2004). On DIV 6, culture medium was removed and replaced with phenol red free DMEM/F12 containing either 0 or 50 mM ethanol for 2 hours at 37 °C. During the last 30 minutes of the treatments, H$_2$DCF-DA (10 µM) was added. The cells were washed once with warm HBSS with Ca$^{2+}$/Mg$^{2+}$ (Invitrogen, Carlsbad, CA) and cells were mounted in HBSS for imaging. Fluorescent images were typically taken within 1 minute after mounting using a fluorescent microscope (Nikon, Tokyo, Japan).
Statistical Analyses

Statistical analyses of the control and ethanol data obtained using the microplate reader were analyzed using a T-test. A p value < .05 was considered to be significant.

Results

The fetal rhombencephalic neurons express 5-HT$_{1A}$ receptors

Because these studies involve the treatment of fetal rhombencephalic neurons with the 5-HT$_{1A}$ agonist ipsapirone, it was important to demonstrate that the cultures of fetal rhombencephalic neurons contain the 5-HT$_{1A}$ receptors. Immunohistochemistry was used to demonstrate that the 5-HT$_{1A}$ receptor was present both on cell bodies and axons (Figure 11).

The fluorescent dye H$_2$DCF-DA is sensitive to changes in intracellular ROS

The fluorescent dye H$_2$DCF-DA is an intracellular ROS detector (Figure 12) and has been used in various cell types (Post et al., 1998; Xu et al., 2004; Zhang et al., 2001). Previously, the use of this dye was not evaluated in live neuronal cultures maintained in low serum, such as that used in the present investigation.

Several experiments were performed to optimize assay conditions and to establish that the DCF method could be used to detect changes in intracellular ROS levels in fetal rhombencephalic neurons. In Figure 13, fluorescence intensities of two selected neurons were measured over time using a confocal microscope. As soon as hydrogen peroxide was added, fluorescence intensities steadily increased over 100 seconds. In other experiments, a fluorescent microplate reader was used to assess relative levels of ROS
over a longer period of time (Figure 14). Fluorescence microscopy was suitable for a rapid qualitative observation, whereas the fluorescent microplate reader allowed quantitative data collection over a longer interval. Both methods confirmed that H$_2$DCF-DA is sensitive to detect ROS.

**Ethanol causes a dose- and time-dependent ROS increase**

H$_2$DCF-DA was used to measure the effects of ethanol on ROS levels in fetal rhombencephalic neurons. After the addition of ethanol the relative fluorescence intensity increases in a dose-dependent manner over an ethanol concentration range of 10 to 50 mM. The increase continues for at least 30 minutes (Figure 15). In comparison to control conditions, all three concentrations of ethanol (10, 25, 50 mM) increased intracellular ROS. Values from ethanol treated cultures were significantly elevated at $p < .05$ or $p < .01$. In addition, the ROS levels increased significantly ($p < .05$) between 10 minutes and 30 minutes after the addition of 50 mM. Subsequent studies used the 50 mM ethanol dose because it caused an increase of ~50% at 30 minutes, and it is the concentration used in previous studies from this laboratory. Due to procedural difficulties in live-cell assays, the changes in intracellular ROS could not be measured beyond 2 hours even with microplate reader. Limited numbers of cells with normal neuronal morphology were identified after 2 hours of assay. Additionally, volatility of ethanol posed further limits.
Co-treatment with ipsapirone does not attenuate the acute ethanol-associated rise in ROS

When ipsapirone and ethanol were added simultaneously, ROS levels increased significantly in both groups and there was no significant difference in ROS levels in the groups treated with ethanol and those co-treated of ipsapirone and ethanol (Figure 16).

Pre-treatment with ipsapirone prevented the ethanol-induced ROS increase

In order to determine whether a longer ipsapirone treatment might help combat the effects of ethanol, cultures of fetal rhombencephalic neurons were pre-treated with ipsapirone for 24 hours before the addition of ethanol. Relative ROS levels were then assessed for 2 hours. A qualitative comparison of relative ROS levels indicated that the 24-hour pre-treatment with ipsapirone prevented an increase in ROS that would otherwise have occurred following a 2-hour ethanol exposure (Figure 17). Unpublished findings from Roberta Gillespie in this laboratory also showed that a 24-hour pre-treatment with ipsapirone significantly prevented the ethanol-induced ROS increase (Figure 18).

Discussion

\(\text{H}_2\text{DCF-DA} \) is sensitive to ROS changes

\(\text{H}_2\text{DCF-DA} \) is one of the most widely used fluorescent dyes to measure changes in intracellular ROS. That it can be applied to both live cells and fixed cells makes \(\text{H}_2\text{DCF-DA} \) very versatile. Detectable changes in relative fluorescence were detected after treatment with hydrogen peroxide, which is both a ROS generator and a major ROS. These changes indicated that \(\text{H}_2\text{DCF-DA} \) can be used to study the acute effects ethanol
on intracellular ROS in primary cultures of fetal rhombencephalic neurons (Figures 13 and 14). It should be mentioned that the decreasing slope of the curve in hydrogen peroxide treatment in Figure 14 might indicate that rapid death of neurons slowed the production of ROS.

A slow increase in ROS over time was observed in cultures of control neurons (no ethanol/no hydrogen peroxide) even though the relative levels of ROS were significantly lower (p > .05) than those in cultures of peroxide- or ethanol-treated neurons (Figure 14). This increase can be explained in two ways. 1) In order to use H$_2$DCF-DA to measure ROS changes, it was necessary to maintain neurons in a media that lacked phenol red and supplements, which would have interfered with the detection of fluorescence in the cultures. Thus, they were maintained in a stressful environment, i.e., one that contained PBS, HBSS, or DMEM/F12 without phenol red and supplements as control media. 2) Cultured primary neurons produce measurable ROS over time as part of normal respiration.

**Ethanol treatment caused a progressive accumulation of ROS**

Evidence from these and additional studies from this and other laboratories show that the effects of ethanol on developing CNS may be caused by oxidative stress (Augustyniak and Skrzydlewska, 2009; Heaton et al., 2003c; Perez et al., 2006). Oxidative stress occurs when the tightly balanced cellular redox status of cells shifts such that ROS levels increase. This can happen when cellular conditions lead to an upsurge of ROS (Augustyniak and Skrzydlewska, 2009) and/or a reduction in endogenous antioxidants (Naziroglu et al., 2003). In the acute ethanol treatment model, it is likely
that oxidative stress results from a rapid increase of ROS within the cell. As previously discussed in Chapter 2, a quick ROS increase after ethanol exposure can be caused by mitochondrial dysfunction via inhibition of electron transport (Bailey et al., 1999; Kayser et al., 2003).

Consistent with findings from the present study, which demonstrated that acute ethanol exposure quickly increased the generation of ROS in cultures of fetal rhombencephalic neurons, another laboratory showed that in vitro acute ethanol treatment resulted in high levels of ROS within 30 minutes in the developing cortical neurons (Ramachandran et al., 2003). In the present study, 50 mM ethanol caused a progressive elevation of ROS over time. The difference between the relative fluorescence in cultures of 50 mM ethanol-treated neurons at 10 and 30 minutes was significant (Figure 15). This is indicative of continuous generation of ROS resulting in ROS accumulation. Without adequate detoxification, this accumulation will lead to significant oxidative neuronal damage.

Effects of ipsapirone on the ethanol-induced increase in ROS

Previous work from other laboratories showed that treatment with one of several classical antioxidants can reduce the ethanol-associated oxidative stress (Guo et al., 2007; Pirlich et al., 2002) and provide neuroprotection (Heaton et al., 2000; Herrera et al., 2003). Most of the classical antioxidants are characterized by their ability to rapidly scavenge free radicals or other ROS. Thus, a reduction of ROS after ethanol exposure should accompany an acute co-treatment with any drugs that have the ROS scavenging ability. In fact, previous studies (unpublished) from this laboratory showed rapid free-
radical scavenging effects by the antioxidant α-lipoic acid after ethanol was added to a culture of fetal rhombencephalic neurons.

The observation from this study that ipsapirone co-treatment did not prevent the ethanol-associated ROS increase (Figure 16) indicates that ipsapirone does not possess the characteristics of a classical free radical scavenging antioxidant. However, a 24-hour ipsapirone pre-treatment attenuated the ethanol-associated increase in ROS (Figure 17 and 18), suggesting that ipsapirone exerts indirect antioxidant properties. Ipsapirone might activate downstream regulators of the levels or activity of endogenous antioxidant enzymes such as SOD1, SOD2, and catalase. For example, ipsapirone might alter the expression of the gene encoding an antioxidant enzyme, the translation of the message into protein, or the stability/turnover of the protein product. The next chapter describes the effects of ipsapirone on the expression and activities of the three noted antioxidant enzymes.

Even though the mechanism is unclear, non-scavenging agents have been identified that reduce oxidative stress by regulating the expression of endogenous antioxidant enzymes. In fact, there are number of drugs shown to help reduce oxidative stress, although the drugs themselves are not direct scavengers of ROS. Instead, they help facilitate the cellular environments where redox balance is well maintained (Dong et al., 2008; Ognjanovic et al., 1995; Warner et al., 1991). Interestingly, the study has demonstrated that the protective mechanism associated with 5-HT₁A receptor in part included attenuation of oxidative stress caused by hydrogen peroxide (Lee et al., 2005b).

Transcription factors like NF-κB, AP-1, and Nrf-2 are likely to regulate the expression of the genes that encode endogenous antioxidant enzymes (Dong et al., 2008;
Pinkus et al., 1996; Rojo et al., 2004). That ipsapirone’s ability to block an ethanol-associated increase in ROS required a 24-hour pre-treatment (Figure 17 and 18) is consistent with the possibility that its effects were exerted by regulation of gene transcription, because both transcriptional changes and subsequent translation require time. In the studies described in subsequent chapter, the effects of ipsapirone on the expression of endogenous antioxidant enzymes were examined.
Figure 11. Serotonin-1A receptors are expressed on cell bodies and axons of the fetal rhombencephalic neurons. Primary neuronal culture of G14 fetal rhombencephalon on DIV5 (A and B) were fixed in 4% paraformaldehyde followed by immunostaining with 5-HT_{1A} primary antibody (B) or without (A). DAB staining was used to visualize 5-HT_{1A} receptors found on cell bodies (arrow heads) and axons (arrows) of the neurons.
Figure 12. Schematic illustration of the intracellular ROS detection fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA). The H$_2$DCF-DA molecule is a membrane permeable fluorescent dye. The diacetate moiety on the molecule allows H$_2$DCF-DA to permeate the cell membrane. Once it enters the intracellular space, cellular esterase hydrolyzes the acetate moiety making the molecule lipophobic HDCF. In the presence of ROS, HDCF get oxidized and becomes fluorescent DCF. Fluorescence intensity is correlated with the amount of intracellular ROS, e.g. superoxide, hydrogen peroxide, and hydroxyl radicals.
Figure 13. **H$_2$DCF-DA detects ROS in primary cultures of fetal rhombencephalic neurons using confocal imaging.** The relative levels of reactive oxygen species (ROS) were determined using 50 µM 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA). Using confocal microscopy, the fluorescence intensity of two marked neurons was recorded over time. (A) Still shots of fetal rhombencephalic neurons exposed to 50 µM of H$_2$O$_2$ over a 30 second interval. (B) A graph depicting fluorescence intensity of the two neurons measured over time.
Figure 14. The H$_2$DCF-DA is useful to detect relative ROS levels in primary culture of fetal rhombencephalic neurons using a fluorescence microplate reader. The relative levels of reactive oxygen species (ROS) were determined using 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) dye. A fluorescence microplate reader was used to detect the fluorescence intensity of the neurons after the addition of PBS (Control) or hydrogen peroxide (H$_2$O$_2$ 200 µM) over a 60 minute interval. Values that are significantly different from the time-matched control value at $p < .01$ are represented by the **. 
Figure 15. Ethanol induces a dose-dependent and time-dependent increase in reactive oxygen species in fetal rhombencephalic neurons. The relative levels of reactive oxygen species (ROS) was determined using 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) dye. Fluorescence intensity was measured after the addition of ethanol (10, 25, 50 mM) over a 30-minute interval. (A) A scatter plot with measurements at 5-minute intervals over a period of 30 minutes. (B) A bar graphic representation of relative fluorescence at 10, 20, and 30-minutes. Values that are significantly different from the time-matched control value at p < .05 and p < .01 are represented by the * and **, respectively.
Figure 16. Co-treatment with ipsapirone did not reduce the ethanol-induced ROS increase in fetal rhombencephalic neurons. The generation of reactive oxygen species (ROS) was determined using 50 µM 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) dye. Fluorescence intensity was measured after the addition of PBS (Control), 50 mM ethanol (Ethanol), 100 nM ipsapirone (Ipsapirone), and ethanol and ipsapirone (Ipsapirone + Ethanol) at the same time and over a 30-minute interval. The results show that co-treatment with ipsapirone did not prevent ethanol-induced ROS increase.
Figure 17. A 24-hour pre-treatment with ipsapirone prevents the ethanol-associated increase in ROS. The generation of reactive oxygen species (ROS) was determined using 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) dye. Ipsapirone treatment was initiated 24 hours prior to the addition of ethanol. The fluorescent images were captured from cultures that were maintained in HBSS for 2 hours (Control 2-Hour) or in cultures that were treated with 50 mM ethanol for 2 hours (Ethanol 2-Hour) or pre-treated with ipsapirone for 24 hours before the addition of ethanol (Pre-Ipsapirone 24-Hour + Ethanol 2-Hour). Pre-treatment with ipsapirone prevented ethanol-induced ROS increase.
Figure 18. A 24-hour pre-treatment with ipsapirone prevents the ethanol-associated increase in ROS in fetal rhombencephalic neurons. The generation of reactive oxygen species (ROS) was determined using 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) dye. Ipsapirone treatment was initiated 24 hours prior to the addition of ethanol. The relative fluorescence was measured using a fluorescence plate reader after cultures were maintained for 30 minutes in PBS (Control) or 50 mM ethanol (Ethanol and Pre-Ipsapirone + Ethanol). Values that are significantly different from control and ethanol value at $p < .05$ are represented by the * and #, respectively.
CHAPTER 4

EFFECTS OF ETHANOL AND IPSAPIRONE ON THE EXPRESSION OF GENES ENCODING ANTIOXIDANT ENZYMES IN ETHANOL-TREATED NEURONS

Abstract

The preceding chapter showed that ethanol increased ROS, while simultaneous treatment with ipsapirone did not prevent the increase in fetal rhombencephalic neurons. However, 24-hour pre-treatment with ipsapirone prevented the ethanol-associated increase in ROS. The findings suggest ipsapirone-associated ROS reduction might involve transcriptional activation of endogenous antioxidants. Previous findings from this laboratory demonstrated that a 24-hour co-treatment with the serotonin-1A agonist ipsapirone prevented ethanol-associated apoptosis and activated the expression of specific NF-κB dependent anti-apoptotic genes, *Xiap* and *Bcl-XL*, in ethanol-treated fetal rhombencephalic neurons (Druse et al., 2004; Druse et al., 2006). The present study investigated: 1) the temporal effects of both ethanol and ipsapirone on the expression of *Sod1*, *Sod2*, and *catalase*, which encode for endogenous antioxidants enzymes and 2) the effects of the SOD/catalase mimetic EUK-134 on the prevention of ethanol-induced apoptosis. Enzyme activities of superoxide dismutase and catalase were also measured. Expression of *Sod2* and *catalase* demonstrated an early activation by ethanol. After a brief treatment with 50 mM ethanol, i.e., 2-hour and 8-hour, *Sod2* and *catalase* were
significantly increased, respectively, possibly as an initial attempt to survive. An ethanol-associated increase in catalase was followed by a rise in catalase activity. Both catalase expression and activity returned to levels found in unstressed controls. A late activation, i.e. 24-hour, of Sod1 and Sod2 by ethanol was detected. Total SOD enzyme activity resembled the changes seen in Sod1 gene expression. Importantly, treatment with ipsapirone augmented the activity of catalase and the expression of catalase in ethanol-treated neurons at later time points. The latter effects are likely to contribute to the pro-survival effects of ipsapirone. EUK-134 prevented ethanol-induced apoptosis in a dose-dependent manner, suggesting the importance of the endogenous antioxidants in the prevention of ethanol toxicity in developing neurons.
INTRODUCTION

*In utero* ethanol exposure can cause serious functional and structural abnormalities in the brain of the developing fetuses. Included among the CNS disorders associated with Fetal Alcohol Syndrome (FAS) and/or Fetal Alcohol Spectrum Disorder (FASD) are problems with attention, behavior, cognition, memory, and executive function (Mattson et al., 1996; Riley et al., 2003; Wattendorf and Muenke, 2005). In addition, morphological abnormalities are found in several brain regions, including the corpus callosum, cerebellum, and basal ganglia (Riley et al., 2004; Roebuck et al., 1998).

Although the mechanism(s) by which alcohol exposure effects the deleterious changes in the developing brain have not been fully elucidated, both *in vivo* and *in vitro* animal studies suggest that apoptosis is likely to be involved (Castoldi et al., 1998; Cheema et al., 2000; Ikonomidou et al., 2000; Ramachandran et al., 2001).

Earlier studies from this (Druse et al., 1999; Tajuddin and Druse, 2001) and another laboratory (Sari and Zhou, 2004) showed that *in utero* ethanol exposure caused a significant reduction in serotonin (5-HT) neurons. Using a 24 hour treatment of fetal rhombencephalic neurons with 50 mM ethanol, *in vitro* studies established that this reduction was probably caused by ethanol-associated apoptosis (Druse et al., 2005; Druse et al., 2004; Druse et al., 2007), a decreased activity of the phosphatidylinositol 3-kinase (PI3K) \( \rightarrow \) pAkt pro-survival pathway (Druse et al., 2005) and reduced downstream expression of several NF-κB dependent anti-apoptotic genes: *Xiap, Ciap1, Ciap2, Bcl-2* and *Bcl-xl* (Druse et al., 2006; Druse et al., 2007). Importantly, *in vivo* and *in vitro* treatment with the 5-HT<sub>1A</sub> agonist ipsapirone prevented the ethanol-associated reduction of 5-HT and other fetal rhombencephalic neurons and the ethanol-associated decrease of
pAkt (Druse et al., 2005; Druse et al., 2004; Tajuddin and Druse, 1999; Tajuddin and Druse, 2001). Ipsapirone was able to increase expression of NF-κB dependent genes that encode XIAP and Bcl-XL in fetal rhombencephalic neurons treated with ethanol for 24 hours prior to the addition of ipsapirone (Druse et al., 2006).

One way by which ethanol augments apoptosis is by increasing oxidative stress. In fact, several laboratories, including this one, have shown that ethanol increases reactive oxygen species (ROS) in developing neural tissue (Heaton et al., 2002; Ramachandran et al., 2003; Watts et al., 2005). The increased oxidative stress is associated with augmented apoptosis (Ramachandran et al., 2003), and co-treatment of fetal rhombencephalic neurons with specific antioxidants prevents ethanol-associated apoptosis (Antonio and Druse, 2008). Antioxidant treatment also prevents damage to ethanol-exposed cerebellar granule cells (Heaton et al., 2004b; Siler-Marsiglio et al., 2004) and hippocampal neurons (Marino et al., 2004), although it has not proved neuroprotective in all studies (Grisel and Chen, 2005; Tran et al., 2005).

Of particular interest to the current study are the antioxidant enzymes SOD1, SOD2, and catalase; these are endogenous antioxidants that participates in reactions that detoxify ROS. These enzymes are of interest both because of their roles as antioxidant enzymes and because there is evidence that Sod1, Sod2, and catalase are expressed in a NF-κB dependent manner (Furukawa et al., 1997; Rojo et al., 2004; Zhou et al., 2001a). Interestingly, catalase is also reportedly the key enzyme involved in ethanol metabolism in the brain of rodents (Zimatkin et al., 2006). Considering that the 5-HT1A agonist ipsapirone upregulates the NF-κB genes encoding the anti-apoptotic proteins Bcl-XL and XIAP (Kucharczak et al., 2003), this drug might also augment the expression of the three
endogenous antioxidants; such an effect could be essential to reducing the ROS-mediated apoptosis caused by ethanol.

In order to better understand the contribution of potential neuroprotective effects of ipsapirone, this study evaluated the time course associated with the effects of ipsapirone and ethanol the genes that encode the antioxidant enzymes SOD1, SOD2, and catalase. This study also examined the effects of ethanol and ipsapirone on total SOD and catalase activity.

MATERIALS AND METHODS

In vitro fetal rhombencephalic neuronal cultures

The methods used in this study have been described previously in several earlier publications from this laboratory (Druse et al., 2005; Druse et al., 2004; Druse et al., 2006; Druse et al., 2007). All animal care and use procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Loyola University Chicago, Stritch School of Medicine. The brain region that contains the developing 5-HT neurons (Konig et al., 1988), i.e., the fetal rhombencephalon, was removed (Eriksen et al., 2002) from timed-pregnancy Sprague-Dawley rats (175-185 grams) at gestation day 14 (G14), where G=0 corresponds to the day of insemination. Throughout the dissection procedure, tissue samples were kept ice-cold in Hank’s balanced buffered solution (HBSS), and sterile instruments and techniques were used. Rhombencephalic tissue was mechanically disaggregated before seeding onto poly-D-lysine coated plates (Corning, Corning, New York) at a density of 8x10^6 cells/plate (55 cm²). Cultures were maintained in a neuron-specific chemically modified media (CMM) that included Dulbecco’s
Minimal Essential Media/F12 (DMEM/F12) media (Invitrogen, Carlsbad, CA), hydrocortisone-21 sulfate (Sigma, St. Louis, MO), Basal Medium Eagle Vitamin Solution (Sigma, St. Louis, MO), antibacterial agent gentamicin sulfate (Honegger and Monnet-Tschudi, 1997), B27 serum-free medium supplement (Invitrogen, Carlsbad, CA) (Brewer et al., 1993), and 0.25% fetal bovine serum (FBS) (Druse et al., 2004). After 24 hours, 0.4 µM cytosine arabinoside (Sigma, St. Louis, MO) was added to arrest gliogenesis. Immunohistochemistry was performed using an antibody to glial fibrillary acidic protein (GFAP) to assess the contribution of astrocytes to the cultures; this method confirmed that astrocytes comprised < 5% of the cells in these neuronally-enriched cultures. Cells were grown in control media (no ethanol) for 5 days, with media changes made on alternate days.

**Treatments**

The fetal rhombencephalic neurons were cultured until DIV 5 according to the method described previously. Using an ethanol chamber system described by this laboratory (Druse et al., 2004), cultures were divided into four groups for RNA isolation and enzyme activity assays: Control (no ipsapirone/ no ethanol), Ipsapirone (100 nM ipsapirone), Ethanol (50 mM ethanol), and Ethanol + Ipsapirone (50 mM ethanol + 100 nM ipsapirone). For the Ethanol + Ipsapirone group, treatment was employed at the same time (co-treatment). The concentration of ipsapirone used in this project was found to promote the development of 5-HT neurons (Whitaker-Azmitia et al., 1990) and prevent apoptosis in fetal rhombencephalic neurons (Druse et al., 2004).
RNA Isolation and cDNA Synthesis

Neurons were treated for different periods, ranging from 2 hours to 48 hours. Trizol reagent (Life Technology, Gaithersburg, MD) (Druse et al., 2006; Druse et al., 2007; Lee et al., 2009) was used to extract total RNA from cultured fetal rhombencephalic neurons. Glycogen (20 mg/ml) (Ambion, Austin, TX) was included to facilitate the precipitation of RNA and to maximize the yield of RNA. RNA was dissolved in 25 µl DEPC-treated H₂O, which was treated with DNA-free (Ambion, Austin, TX) to remove contaminating genomic DNA. RNA was stored at −80° until use. Single strand cDNA was synthesized from 1 to 2 µg of total RNA (DNA-free) using the First Strand cDNA synthesis kit (Fermantas, Hanover, MD).

Quantitative real-time RT-PCR

cDNA (DNA equivalent of 20 ng to 40 ng of total RNA) was diluted with Platinum Quantitative PCR Super Mix-UDG [1.5 U Platinum Taq DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 mM dGTP, 200 mM dATP, 200 mM dCTP, 400 mM dUTP, 1 U UDG (Life Technology)], 0.25 mM Rox (Life Technology), 1/40,000 SYBR Green (Molecular Probes, Eugene, Oregon), and primers (Druse et al., 2006; Lee et al., 2009). A Perkin-Elmer Gene Amp 7300 Sequence Detector thermal cycler (Applied Biosystems, Foster City, CA) was used for PCR amplifications. RT-PCR data was analyzed using SDS software (Applied Biosystems). Sample inputs were normalized using expression of GAPDH.

Plasmid containing target genes were used to generate standard curves for real-time quantitative RT-PCR assays (Druse et al., 2006), and a standard curve was generated
from serial dilutions of known amounts of the input copy number of target genes. Standard curves for *GAPDH* and genes of interest (10-100,000 copies/µl) were performed in each experiment. Routinely we included a triplicate RT-PCR reaction lacking cDNA or known DNA template for each set of primers. Specific primary sequences for *Sod1*, *Sod2*, *catalase* and *GAPDH* were selected using the Primer Express program (Applied Biosystems, Foster City, CA) and sequences available from the NCBI database. Primers were synthesized by Invitrogen (Carlsbad, CA). The forward (F) and reverse (R) primer sequences for rat genes are included in Table 2. Typically, *GAPDH* and the three target genes were first detected at 22 or 28 cycles, respectively. As previously described (Druse et al., 2006; Druse et al., 2007) data was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

**Superoxide dismutase activity assay**

Neurons were treated for different periods, ranging from 2 hours to 48 hours. All the treatment groups were harvested according to the manufacturer’s protocol provided in the assay kit (Cayman Chemical, Ann Arbor, MI). Treated cells were first washed twice with ice cold 1X PBS (pH 7.4) and collected in 300 uL of ice cold lysis buffer [20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose] using a rubber policeman. Harvested cells were sonicated followed by centrifugation at 1,500 x g for 5 minutes at 4°C. The supernatant was removed and stored at -80°C until the day of the assay.

On the day of the assay, all the reagents provided in the assay kit were prepared according to the manufacturer’s protocol. The frozen samples were thawed in ice and
processed quickly on ice. Once all the samples and reagents were prepared in the 96-well plate, the absorbance was read at 460 nm using a plate reader (Molecular Devices, Sunnyvale, CA). Subsequently, total protein concentration for each sample was determined using Lowry’s protein assay (Lowry et al., 1951). Using the calculation formula provided by the manufacturer’s protocol, the superoxide dismutase enzymatic activity was determined based on the total amount of the protein in each sample.

**Catalase activity assay**

Neurons were treated for different periods, i.e., 18, 24, or 48 hours. The activity of catalase was determined using an enzymatic kit (Cayman, Ann Arbor, MI). After treatment, the treatment groups were harvested according to the manufacturer’s protocol provided in the assay kit (Cayman Chemical, Ann Arbor, MI). Treated cells were first washed twice with ice cold 1X PBS (pH 7.4) and collected in 300 uL of ice cold lysis buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) using a rubber policeman. Harvested cells were sonicated followed by centrifugation at 10,000 x g for 15 minutes at 4°C. The supernatant was removed and stored at -80°C until the day of the assay.

On the day of the assay, all the reagents provided in the assay kit were prepared according to the manufacturer’s protocol. The frozen samples were thawed in ice and processed quickly on ice. Once all the samples and reagents were prepared in a 96-well plate, the absorbance was read at 540 nm using a plate reader (Molecular Devices, Sunnyvale, CA). Subsequently, total protein concentration for each sample was determined using Lowry’s protein assay (Lowry et al., 1951). Using the calculation
formulas provided by the manufacturer’s protocol, the catalase enzymatic activity was determined based on the total amount of the protein in each sample.

**Lowry’s protein assay**

To determine the total protein concentration Lowry’s protein assay was used (Lowry et al., 1951). First, 10 µL of a sample was transferred to a glass test tube. Also, standard protein concentrations were prepared with 5, 10, 20, 30, 40 µL of bovine serum albumin (1 mg/mL). To each tube, 100 µL of 1N NaOH was added and mixed followed by 30 minutes incubation at 25°C. Then, 1 mL of phenol was added to each tube and incubated for 30 minutes at 25°C. Finally, 100 µL of phenol reagent was added to each tube for 30 minutes at 25°C followed by absorbance reading at 595 nm.

**EUK-134 treatment and Hoechst 33342 staining**

Primary neuronal cultures of fetal rhombencephalon were treated with 0 or 50 mM ethanol and EUK-134 (0, 0.5 µM or 5 µM) for 24 hours. Treated cells were then stained with Hoechst 33342 as previously described (Antonio and Druse, 2008; Druse et al., 2005).

**Statistical analyses**

Data was analyzed using two-way ANOVA and a post-hoc Newman-Keuls or Dunnet’s procedure to test our hypotheses that ethanol treatment alters the expression of some NF-κB dependent genes that encode antioxidant enzymes and that ipsapirone
restores expression in ethanol-treated neurons to levels found in untreated (control) cultures. A p value < .05 was considered to be significant.

RESULTS

Effects of ethanol and ipsapirone on Sod1

At 2 hours of treatment, ipsapirone significantly increases Sod1 in control (no ethanol) neurons and reduces it in ethanol-treated neurons (p<.05) (Figure 19). In addition, ethanol significantly elevated Sod1 in neurons that were maintained under control conditions or co-treated with ipsapirone for 24 hours (p < .01) (Figure 20). However, by 48 hours there was no longer an ethanol-associated increase in Sod1 (Figure 21). Values in the ethanol group returned to control levels by this time, and those in the ethanol plus ipsapirone group were lower than those in control (no ethanol neurons) (p < .05).

Effects of ethanol and ipsapirone on Sod2

At the 2-hour time point, ipsapirone augmented expression of Sod2 in both control and ethanol-treated neurons (Figure 24). Ethanol treatment also increased Sod2 at this early time. At 24 hours, ethanol augmented Sod2 in cultures maintained in the presence and absence of ipsapirone (p < .05) (Figure 25). This increase was maintained until 48 hours in the Ethanol plus Ipsapirone group (Figure 26), but Sod2 returned to control levels in neurons treated only with ethanol. Although it appears that Sod2 was elevated in control cultures at 48 hours (Figures 27 & 28), the difference was not significant (p > .05).
Effects of ethanol and ipsapirone on SOD activity

There were only two times when the activity of total SOD activity was affected by either ethanol or ipsapirone (Figure 31). At 2 hours (Figure 29) there was an ipsapirone-mediated increase in SOD activity (p < .05), and there was a significant decrease at 48 hours (Figure 30) in the ethanol plus ipsapirone treatment group (p < .05). At the other times examined, the enzyme activities in the treatment groups were comparable to those in control neurons. Because of the low level of activity of the separate enzymes, total SOD activity was determined.

Expression and activity changes in catalase

Treatment of fetal rhombencephalic neurons with 50 mM ethanol caused a modest yet significant increase (p < .05) in catalase at 8 hours (Figure 32). This early elevation was brief and transient. This increase in gene expression was followed by an elevation of catalase activity at 18 hours (p < .05) (Figure 36). A prolonged (24 hour) exposure to ethanol brought catalase levels down to those in unstressed controls. Importantly, catalase was significantly elevated in Ethanol plus Ipsapirone-treated cultures (Ethanol + Ipsapirone group versus Ethanol group) at 24 hours (p < .05) (Figure 33). Even when compared to control neurons, ipsapirone treatment caused a significant increase in catalase at 24 hours. It should be noted that addition of 100 nM ipsapirone to control neurons (Ipsapirone group) significantly augmented the expression of catalase (Figure 33) at 24 hours (p < .01) when compared to control neurons. Catalase enzyme activity was increased only in the co-treatment group at 48 hours (Figure 37).
EUK-134 protects against ethanol-mediated apoptosis

When fetal rhombencephalic neurons were treated with ethanol for 24 hours, ~25%-30% underwent apoptosis. However, co-treatment with EUK-134, a SOD/catalase mimic, significantly reduced ethanol-associated apoptosis. This was found when neurons were co-treated with either 0.5 µM (p < .05) or 5 µM (p < .01) of EUK-134. However, only the higher dose of EUK-134 fully blocked ethanol-associated apoptosis.

DISCUSSION

Early and transient response to ethanol

Ethanol treatment caused an early and significant increase in the expression and activities of SOD (Figure 22, 23, 27, 28, and 31) and catalase (Figure 34, 35, and 38). The present study found an ethanol-associated increase in the expression of the gene encoding the antioxidant enzymes SOD2 (2 hours) and catalase (8 hours). Although the ethanol-associated change in Sod2 was not paralleled by a similar change in enzyme activity, the significant increase in catalase was followed by a corresponding elevation in catalase activity. Thus, it appears that ethanol causes an early activation of two genes that encode antioxidant enzymes and that one of these changes resulted in a change in enzyme activity.

Other investigators report that ethanol augmented the activity and/or expression of these antioxidant enzymes. For example, ethanol treatment elevated SOD activity in the developing striatum, hippocampus, and cortex (Enache et al., 2008; Heaton et al., 2003b; Heaton et al., 2003c). In addition, an acute in vivo ethanol exposure resulted in an early increase in catalase (Heaton et al., 2003a; Reddy et al., 1999). Ethanol-associated up-
regulation of catalase and Sod2 was also detected in hepatic cells (Bardag-Gorce et al., 2006; Heaton et al., 2003c). Moreover, additional studies from this laboratory showed that the early (2 to 8 hour) response of fetal rhombencephalic neurons to 50 mM ethanol correlates with augmented expression of two pro-survival genes (i.e., Xiap, Bcl-xl) (Lee et al., 2009).

The significance of an early ethanol-mediated up-regulation of genes encoding antioxidant enzymes and/or anti-apoptotic factors may represent an attempt of fetal rhombencephalic neurons to defend themselves against the pro-apoptotic effects of ethanol. For example, XIAP is an inhibitor of the apoptotic enzyme caspase-3 (Salvesen and Duckett, 2002), and Bcl-xl would stabilize the mitochondria, the epicenter of ethanol-mediated cell damage, from releasing cytochrome C. Moreover, increased mitochondrial SOD2 stabilizes mitochondria by neutralizing $O_2^{-}$ to hydrogen peroxide, which is further reduced to water by catalase (Song and Lee, 2003; Temple et al., 2005; Wheeler et al., 2001). Therefore, SOD and catalase could potentially attenuate ethanol-associated oxidative stress, i.e., $O_2^{-}$ and hydrogen peroxide production, and the associated apoptosis. Alternatively, it is possible that the ethanol-mediated early increase in catalase might contribute to the neuron’s efforts to metabolize ethanol. However, the toxic product, acetaldehyde, is not further metabolized, this could damage the developing brain (Lee et al., 2005).

Although the mechanism by which ethanol exposure initially increased the expression of Sod2 and catalase genes has not been confirmed, it could involve the rapid rise in ROS which accompanies ethanol exposure (Ramachandran et al., 2003; Watts et al., 2005). An increase in cellular ROS can activate several classes of genes, including
those that are dependent on NF-κB (Ji et al., 2004; Turpaev, 2002; Zhou et al., 2001a) and Nrf2 (Zhu et al., 2005), which binds to the antioxidant response element (ARE) [reviewed by (Kensler et al., 2007)]. Catalase as well as XIAP and Bcl-xl can be regulated by NF-κB dependent mechanisms (Kucharczak et al., 2003; Zhou et al., 2001a).

Catalase and SOD also can be regulated Nrf2 (Zhu et al., 2005). Thus, it is likely that the early ethanol-associated augmentation of gene expression is mediated by increased ROS on ROS-sensitive transcription factors. Consistent with this explanation, microarray studies show that ethanol upregulates additional CNS genes that are known to be induced by oxidative stress (Treadwell and Singh, 2004).

Late response to ethanol

Although the early ethanol-associated rise in catalase and Sod2 are accompanied or followed by an increase in enzyme activity, these potentially protective effects are transient. Expression of Sod1 in ethanol-treated neurons returns to levels in control/unstressed neurons by 48 hours; catalase falls by 24 hours. In both cases, enzyme activity was comparable to levels in unstressed neurons. In fact, with the exception of the mentioned early rise and a later rise of Sod1 and Sod2 expression (but not activity) at 24 hours, the expression of the Sod and catalase genes and the associated enzyme activities was comparable to that in unstressed/control neurons at other times.

Temporal and brain region specific effects of ethanol on the SOD and catalase enzymes were previously reported (Heaton et al., 2003a; Heaton et al., 2003b; Heaton et al., 2003c). Although the levels of antioxidant enzymes at 24 and 48 hours might be suitable for unstressed neurons, it is doubtful they are sufficient to counteract the
damaging effects of oxidative stress. Consequently, the ethanol-treated neurons may not be able to combat the effects of increased ROS for more than a brief time. Although we did not detect an ethanol-associated reduction in SOD activity in our in vitro studies, lower SOD activity was found in brain following in utero exposure to ethanol (Ledig et al., 1981).

We cannot eliminate the possibility that the ethanol-mediated increase in Sod1 and Sod2 at 24 hours is accompanied by augmented activity of SOD at a time between 24 and 48 hours, which was not examined in this study. Such changes could exacerbate the oxidative stress by overproducing hydrogen peroxide. Oxidative stress could develop because the ethanol-treated neurons do not have a corresponding increase in catalase expression or activity at that time, and catalase would be necessary to convert hydrogen peroxide to water.

It is curious that there was an ethanol-associated increase in Sod1 and Sod2 at 24 hours when many cells had already undergone apoptosis. At present, we do not have an explanation for this second rise in the expression of these genes. It is possible that the secondary increase in Sod1 and Sod2 represents another attempt of surviving neurons to resist the damaging effects of ethanol, and that this was mediated by increased ROS. However, such an attempt would have been futile since there does not appear to be a corresponding increase in SOD activity.

This laboratory also reported that an initial ethanol-associated rise in Xiap and Bcl-xl (Lee et al., 2009) is followed by a decline to subnormal levels. Similarly, Ciap1, Ciap2, and Bcl-2 were subnormal after a 24-hour exposure to ethanol (Druse et al., 2006). The similar timing of the reduced expression of pro-survival genes and inappropriately
low expression of genes that encode antioxidant enzymes correlate with augmented apoptosis (Druse et al., 2005; Druse et al., 2004; Druse et al., 2007) and with reduced activity of the PI3K→pAkt pro-survival pathway (Druse et al., 2005).

**Early response to ipsapirone**

As noted previously, ipsapirone prevented the early ethanol-mediated increase in catalase expression. This implies a possible role of ipsapirone to mediate regulation of catalase expression. Because catalase is the major enzyme to metabolize ethanol in rat brain (Zimatkin et al., 2006), an early increase in catalase might augment-ethanol metabolism. Such an effect could help reduce the damaging direct effects of ethanol. However, it would also have the potential to increase toxic acetaldehyde levels, unless this compound were efficiently degraded to acetate (Giavini et al., 1992; Lee et al., 2005).

Although the signaling mechanism by which ipsapirone influences the expression of catalase, Sod1 and Sod2 in control or ethanol-treated neurons is not known, it could involve receptor-mediated activation of the PI3K→pAkt pro-survival pathway, which reportedly regulates the expression of SOD1 (Rojo et al., 2004).

At two time points, 2 and 48 hours, there was a significant interaction between ethanol and ipsapirone, which resulted in decreased Sod1 in the ethanol plus ipsapirone treatment group. The change at the latter time point corresponded with reduced activity of SOD. As noted, SOD is involved with the detoxification of ROS by catalyzing a reaction which forms hydrogen peroxide. Normally, catalase would further detoxify this species of ROS by converting it to water. At present, it is not known whether the reduced
SOD attenuates oxidative stress by lowering the production of hydrogen peroxide or whether it augments oxidative stress by limiting the detoxification of superoxide anion. In addition, the mechanism by which the combined effects are mediated is not clear, but it might involve crosstalk between ethanol-mediated (involving ROS) and receptor-mediated Sod1 expression.

**Late response to ipsapirone**

At later critical time points, ipsapirone exerted potentially neuroprotective effects on ethanol-treated neurons. In fact, at times when ethanol significantly reduced XIAP and/or Bcl-xl, i.e., (16-48 hours), co-treatment with ipsapirone either brought the expression of these genes back to control levels or significantly increased expression above the levels in ethanol-treated or control neurons (Lee et al., 2009). In addition, ipsapirone upregulated catalase expression at 24 hours and activity by 48 hours. Augmented expression of the NF-κB-dependent genes XIAP and Bcl-xl in ethanol-treated neuronal cultures appears to be related to the ability of ipsapirone to increase PI3K → pAkt (Druse et al., 2006), because the PI3K inhibitor LY 294002 blocked the ipsapirone-mediated increase. An investigation of the effects of the PI3K inhibitor on catalase is discussed in a subsequent chapter. It should also be noted that the timing of the ipsapirone-mediated increases in Xiap, Bcl-xl, Sod1, Sod2, and catalase were not identical, suggesting the possibility that multiple transcription factors participate in the regulation of these genes (Ji et al., 2004; Kucharczak et al., 2003; Turpaev, 2002; Zhou et al., 2001a; Zhu et al., 2005).
It is likely that ipsapirone’s ability to block an ethanol-associated decrease in the expression of anti-apoptotic genes or augment such expression is meaningful biologically because ipsapirone treatment also blocked ethanol-associated apoptosis at 24 hours (Druse et al., 2005; Druse et al., 2004). It is unlikely that the ipsapirone-mediated elevation of catalase activity at 48 hours can explain ipsapirone’s anti-apoptotic effects at 24 hours. However, this change could contribute to the neuroprotective effects that are observed when ipsapirone is co-administered with ethanol on a more prolonged basis (> 24 hours). For example, the catalase changes might contribute to ipsapirone-mediated neuroprotection found in vivo when ethanol-treated pregnant dams are treated with this or a related drug during the latter portion of gestation (Tajuddin and Druse, 1999; Tajuddin and Druse, 2001). Consistent with this hypothesis, a 24-hour co-treatment with catalase reduced the ethanol-associated increase in ROS and protected from ethanol-mediated cell death in PC12 cells (Li et al., 2001a).

In contrast to the evidence that ipsapirone’s neuroprotective effects might be linked with the up-regulation of anti-apoptotic/pro-survival genes and increased expression and activity of catalase, it does not appear that changes in Sod1 and Sod2 contribute to these effects. In fact, the only times when there was either a main effect of ipsapirone or an interaction of the effects of ipsapirone and ethanol were at 2 and 48 hours for both SOD genes. However, ipsapirone decreased Sod1 at both times in ethanol-treated neurons. In addition, the ipsapirone-mediated increase in Sod1 in control neurons did not attenuate apoptosis under basal conditions (Druse et al., 2004). Regarding Sod2, both ipsapirone and ethanol increased gene expression at 2 hours and these effects were not additive. Although Sod2 was increased in the ethanol plus ipsapirone group at the 48-
hour time point, SOD enzyme activity was actually reduced at that time. Nonetheless, we cannot rule out the possibility that the increased Sod2 at 48 hours led to an increase in SOD activity at a later time.

Although ipsapirone significantly increased catalase, Sod1, and Sod2 in control neurons, these changes were not followed by increased enzyme activity. Similarly, ipsapirone-mediated increases in the expression of Xiap and Bcl-xl in control neurons did not augment survival in these neurons (Druse et al., 2005; Druse et al., 2004). Evidently, there are cellular compensatory mechanisms in control neurons which prevent overproduction of certain molecules.

Protective effects of EUK-134

Many investigators previously demonstrated the protective effects of EUK-134 against different diseases (Chen et al., 2004; Pong et al., 2001; Rong et al., 1999; Samai et al., 2007). Particularly, EUK-134 prevented limb malformations induced by ethanol exposure during the gestational period (Chen et al., 2004). Evidence that this synthetic SOD/catalase mimetic reduced ethanol-associated apoptosis suggests that ethanol-induced apoptosis is associated with oxidative stress, and that endogenous antioxidant enzymes such as SOD and catalase can exert neuroprotective effects against the damaging effects of ethanol.

It is important to note that there was gradually increasing apoptosis (though not significantly different) in cultures with higher concentrations of EUK-134 even in the absence of ethanol. Such effects might be caused due to antioxidant-mediated pro-
oxidizing cellular status after introducing high concentrations of antioxidants [reviewed in (Halliwell, 2000)].
Table 2. Sequences of primers used.

<table>
<thead>
<tr>
<th>Rat Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>TGGAGTCTACTGGCGGTTCAC</td>
<td>GGCATGGACTGTGGTCATGA</td>
</tr>
<tr>
<td>Catalase</td>
<td>CAGGTGCGGACATTCTATACGA</td>
<td>GCTTTTCCCCTTGGCAGCTATG</td>
</tr>
<tr>
<td>SOD1</td>
<td>AAGAAACATGGCGGTCCAG</td>
<td>GCAATCCCAATCACACCACA</td>
</tr>
<tr>
<td>SOD2</td>
<td>TGGAGGCTATCAAGCCTGACTT</td>
<td>AGCGTGCTCCCACACATCAAT</td>
</tr>
</tbody>
</table>
Figure 19. **Ipsapirone increases Sod1 mRNA in fetal rhombencephalic neurons at 2 hours.** Each value represents the mean ± the SEM of values obtained from 4 to 6 separate experiments. Values are expressed as the fold change in mRNA as calculated by the \(2^{-\Delta\Delta C_T}\) method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at \(p < .05\) are represented by the *. The # identifies values that are significantly different from the time-matched ipsapirone value at \(p < .05\).

Two-way ANOVA (Ethanol x Ipsapirone)
Ethanol x Ipsapirone Interaction: \(F(1,18) = 15.45, p < .01\)
Figure 20. Ethanol and co-treatment increases Sod1 mRNA in fetal rhombencephalic neurons at 24 hours. Each value represents the mean ± the SEM of values obtained from 3 to 4 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .01$ are represented by the **. The ## identifies values that are significantly different from the time-matched ipsapirone value at $p < .01$.

Two-way ANOVA (Ethanol x Ipsapirone)
Ethanol: $F (1,13) = 42.4$, $p < .01$
Figure 21. Co-treatment decreased Sod1 mRNA in fetal rhombencephalic neurons at 48 hours. Each value represents the mean ± the SEM of values obtained from 4 to 5 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ are represented by the *. The # identifies values that are significantly different from the time-matched ipsapirone value at $p < .05$.

Two-way ANOVA (Ethanol x Ipsapirone)
- Ethanol: $F (1,17) = 10.3$, $p < .01$
- Ethanol x Ipsapirone Interaction: $F (1,17) = 4.4$, $p < .05$
Figure 22. The effects of ethanol and ipsapirone on Sod1 mRNA expression over time. Each value represents the mean ± the SEM of values obtained from 3 to 6 separate experiments: Control (clear), Ipsapirone (dotted), Ethanol (dark), and Ethanol + Ipsapirone (gray). Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ and $p < .01$ are represented by the * and **, respectively. The # and ## identifies values that are significantly different from the time-matched ipsapirone value at $p < .05$ and $p < .01$, respectively.

Two-way ANOVA (Ethanol x Ipsapirone)
- 2 hours - Ethanol x Ipsapirone Interaction: $F(1,18) = 15.45$, $p < .01$
- 24 hours - Ethanol: $F(1,13) = 42.4$, $p < .01$
- 48 hours - Ethanol: $F(1,17) = 10.3$, $p < .01$
- Ethanol x Ipsapirone Interaction: $F(1,17) = 4.4$, $p < .05$
Figure 23. Scatter plot showing the effects of ethanol and ipsapirone on Sod1 mRNA over time. Each value represents the mean ± the SEM of values obtained from 4 to 11 separate experiments: Control (flat), Ipsapirone (circle), Ethanol (triangle), and Ethanol + Ipsapirone (square). Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta \Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 and p < .01 are represented by the * and **, respectively. The # and ## identifies values that are significantly different from the time-matched ipsapirone value at p < .05 and p < .01, respectively.
**Figure 24.** Ipsapirone, Ethanol, and co-treatment increased Sod2 mRNA in fetal rhombencephalic neurons at 2 hours. Each value represents the mean ± the SEM of values obtained from 10 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ and $p < .01$ are represented by the * and **, respectively.

Two-way ANOVA (Ethanol x Ipsapirone)
- Ethanol: $F (1,35) = 7.10$, $p < .05$
- Ethanol x Ipsapirone Interaction: $F (1,35) = 8.82$, $p < .01$
Figure 25. Ethanol and co-treatment increased *Sod2* mRNA in fetal rhombencephalic neurons at 24 hours. Each value represents the mean ± the SEM of values obtained from three to 4 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ is represented by the *.

Two-way ANOVA (Ethanol x Ipsapirone)
Ethanol: $F(1,15) = 10.9$, $p < .01$
Figure 26. Co-treatment increased Sod2 mRNA in fetal rhombencephalic neurons at 48 hours. Each value represents the mean ± the SEM of values obtained from 5 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ are represented by the *.

Two-way ANOVA (Ethanol x Ipsapirone)
Ipsapirone: $F (1,18) = 5.13$, $p < .05$
Figure 27. The effects of ethanol and ipsapirone on mitochondrial Sod2 mRNA expression over time. Each value represents the mean ± the SEM of values obtained from 4 to 11 separate experiments: Control (clear), Ipsapirone (dotted), Ethanol (dark), and Ethanol + Ipsapirone (gray). Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta CT}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ and $p < .01$ are represented by the * and **, respectively.

Two-way ANOVA (Ethanol x Ipsapirone)

- 2 hours - Ethanol: $F (1,35) = 7.10$, $p < .05$
  
  Ethanol x Ipsapirone Interaction: $F (1,35) = 8.82$, $p < .01$

- 24 hours - Ethanol: $F (1,15) = 10.9$, $p < .01$

- 48 hours - Ipsapirone: $F (1,18) = 5.13$, $p < .05$
Figure 28. Scatter plot showing the effects of ethanol and ipsapirone on mitochondrial Sod2 mRNA over time. Each value represents the mean ± the SEM of values obtained from 4 to 11 separate experiments: Control (flat), Ipsapirone (circle), Ethanol (triangle), and Ethanol + Ipsapirone (square). Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ and $p < .01$ are represented by the * and **, respectively.
Figure 29. *Ipsapirone increases SOD enzyme activity after 2 hours.* Each value represents the mean ± the SEM of values obtained from 4 to 5 separate experiments. Values are expressed as the fold change in SOD enzyme activity (U/mL•µg). Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *.

**Two-way ANOVA (Ethanol x Ipsapirone)**

Ethanol x Ipsapirone Interaction: F (1,18) = 7.9, p < .05
Figure 30. Co-treatment decreases SOD enzyme activity after 48 hours. Each value represents the mean ± the SEM of values obtained from 4 separate experiments. Values are expressed as the fold change in SOD enzyme activity (U/mL•µg). Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *.

Two-way ANOVA (Ethanol x Ipsapirone)
Ethanol: F (1,15) = 9.13, p < .01
Figure 31. The effects of ethanol and ipsapirone on SOD enzyme activity over time. Each value represents the mean ± the SEM of values obtained from 3 to 6 separate experiments: Control (clear), Ipsapirone (dotted), Ethanol (dark), and Ethanol + Ipsapirone (gray). Values are expressed as the fold change in SOD enzyme activity (U/mL·µg). Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *.

Two-way ANOVA (Ethanol x Ipsapirone)
2 hours - Ethanol x Ipsapirone Interaction: F (1,18) = 7.90, p < .05
48 hours - Ethanol: F (1,15) = 9.13, p < .01
**Figure 32. Ethanol increases catalase mRNA in fetal rhombencephalic neurons at 8 hours.** Each value represents the mean ± the SEM of values obtained from 3 to 5 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ are represented by the *.

Two-way ANOVA (Ethanol x Ipsapirone)
Ipsapirone: $F(1,15) = 8.59$, $p < .05$
Figure 33. Ipsapirone and co-treatment increased catalase mRNA in fetal rhombencephalic neurons at 24 hours. Each value represents the mean ± the SEM of values obtained from 6 to 7 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta Ct}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 and p < .01, are represented by the * and **, respectively. The ¶ and ¶¶ identifies values that are significantly different from those in the ethanol group (p<.05 and p<.01, respectively).

Two-way ANOVA (Ethanol x Ipsapirone)

Ethanol: F (1,24) = 5.75, p < .05
Ipsapirone: F (1,24) = 35.9, p < .01
Ethanol x Ipsapirone Interaction: F (1,24) = 4.31, p < .05
Figure 34. The effects of ethanol and ipsapirone on catalase mRNA over time. Each value represents the mean ± the SEM of values obtained from 3 to 7 separate experiments: Control (clear), Ipsapirone (dotted), Ethanol (dark), and Ethanol + Ipsapirone (gray). Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ and $p < .01$ are represented by the * and **, respectively. The ¶ and ¶¶ identifies values that are significantly different from those in the ethanol group ($p<.05$ and $p<.01$, respectively).

Two-way ANOVA (Ethanol x Ipsapirone)

- 8 hours - Ipsapirone: $F (1,15) = 8.59, p < .05$
- 24 hours - Ethanol: $F (1,24) = 5.75, p < .05$
  - Ipsapirone: $F (1,24) = 35.9, p < .01$
  - Ethanol x Ipsapirone Interaction: $F (1,24) = 4.31, p < .05$
Figure 35. Scatter plot showing the effects of ethanol and ipsapirone on catalase mRNA over time. Each value represents the mean ± the SEM of values obtained from 4 to 11 separate experiments: Control (flat), Ipsapirone (circle), Ethanol (triangle), and Ethanol + Ipsapirone (square). Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .05$ and $p < .01$ are represented by the * and **, respectively. The ¶ and ¶¶ identifies values that are significantly different from those in the ethanol group at $p<.05$ and $p<.01$, respectively.
Figure 36. Ethanol increases catalase enzyme activity after 18 hours. Each value represents the mean ± the SEM of values obtained from 5 to 6 separate experiments. Values are expressed as the fold change in catalase enzyme activity (U/mL•µg). Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *.

Two-way ANOVA (Ethanol x Ipsapirone)
Ethanol: F (1,21) = 4.3, p < .05
Figure 37. Co-treatment increases catalase enzyme activity in ethanol-treated neurons at 48 hours. Each value represents the mean ± the SEM of values obtained from 3 to 5 separate experiments. Values are expressed as the fold change in catalase enzyme activity (U/mL•µg). Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *. The # identifies values that are significantly different from the time-matched Ipsapirone value at p < .05.

Two-way ANOVA (Ethanol x Ipsapirone)
Ethanol: F (1,17) = 11.6, p < .01
Ipsapirone: F (1,17) = 5.9, p < .05
Ethanol x Ipsapirone Interaction: F (1,17) = 4.8, p < .05
Figure 38. The effects of ethanol and ipsapirone on catalase enzyme activity over time. Each value represents the mean ± the SEM of values obtained from 3 to 6 separate experiments: Control (clear), Ipsapirone (dotted), Ethanol (dark), and Ethanol + Ipsapirone (gray). Values are expressed as the fold change in SOD enzyme activity (U/mL•µg). Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *. The # identifies values that are significantly different from the time-matched Ipsapirone value at p < .05.

Two-way ANOVA (Ethanol x Ipsapirone):
18 hours - Ethanol: F (1,21) = 4.3, p < .05
48 hours - Ethanol: F (1,17) = 11.6, p < .01
Ipsapirone: F (1,17) = 5.9, p < .05
Ethanol x Ipsapirone Interaction: F (1,17) = 4.8, p < .05
Figure 39. Chemical structure of EUK-134.
Figure 40. Dose-dependent protection by the SOD/catalase mimetic EUK-134 against ethanol-associated apoptosis. (A) Hoechst 33342 dye shows blue staining of the non-apoptotic and apoptotic nuclei; apoptotic nuclei (condensed or fragmented) are indicated with the arrows. (B) The bar graph shows dose-dependent protection by EUK-134 from ethanol-induced apoptosis in the fetal neurons. Each value represents the mean ± the SEM of values obtained from 5 to 6 separate experiments. Values that are significantly different from the control value at p < .05 and p < .01 are represented by the * and **, respectively. The # and ## identify values in the ethanol + EUK-134 group that are significantly different from those in the ethanol group (p < .05 and p < .01, respectively).

Two-way ANOVA (Ethanol x Ipsapirone)
- Ethanol: F (1,33) = 26.1, p < .01
- Ethanol x Ipsapirone Interaction: F (1,33) = 18.3, p < .01
CHAPTER 5

IPSAPIRONE-ASSOCIATED ENDOGENOUS ANTIOXIDANT GENE REGULATION INVOLVES COMMON PRO-SURVIVAL PATHWAYS

ABSTRACT

The studies described in Chapter 4 demonstrated that ethanol and ipsapirone activate the expression of endogenous antioxidant enzymes, including Cu/Zn-SOD (SOD1), Mn-SOD (SOD2), and/or catalase. The expression of these genes is mediated by the transcription factor nuclear factor-κB (NF-κB) (Rojo et al., 2004; Sompol et al., 2006; Zhou et al., 2001b). Also, previous findings from this laboratory suggest that one of the neuroprotective effects of the 5-HT$_{1A}$ agonist ipsapirone is linked to activation of the phosphoinositide 3-kinase (PI-3K) → pAkt pro-survival pathway and downstream upregulation of NF-κB dependent pro-survival genes (Druse et al., 2006; Lee et al., 2009). There is also evidence that stimulation of the 5-HT$_{1A}$ receptor activates mitogen-activated protein kinase (MAPK) pathway (Adayev et al., 1999), another pro-survival pathway that can activate NF-κB (Abdouh et al., 2004). The current study examined the potential involvement of the PI-3K and MAPK pathways in the ethanol- and/or ipsapirone-induced activation of NF-κB dependent endogenous antioxidant genes, which occurs after two hours of treatment. The results demonstrate that the early/2-hour ipsapirone-associated upregulation of Sod1 was inhibited by both LY294002 and PD98059, inhibitors of PI-3K and MAPKK, respectively. Upregulation of Sod2 by
ethanol and/or ipsapirone at 2 hours was also inhibited by the two inhibitors. Thus, it appears that the augmented expression of the genes that encode NF-κB dependent endogenous antioxidant enzymes by ethanol and/or ipsapirone is mediated by the PI-3K and MAPK pro-survival pathways.
INTRODUCTION

Two major signaling pathways that exert pro-survival signals and provide protection against cell death, include the PI-3K and MAPK pathways. Ipsapirone-mediated activation of the PI-3K pathway protected developing neurons against ethanol toxicity (Druse et al., 2005). The MAPK pathway is also linked with promoting survival (Adayev et al., 1999). Importantly, serotonin-1A agonists activate both of these pro-survival signaling pathways (Adayev et al., 1999; Cowen et al., 1997; Hsiung et al., 2005). In addition, the protective effects of vitamin E against neuronal oxidative stress and cell death also appear to involve the activation of both pathways (Numakawa et al., 2006). Upon activation, these pathways can converge at one common downstream effector that is subsequently activated to mediate a pro-survival signal. Transcription factor NF-κB could be such a signal-converging downstream effector, because both the MAPK and PI-3K pathways activate NF-κB (Dhandapani et al., 2005; Norris and Baldwin, 1999; Schouten et al., 1997). Thus, activation of the 5-HT1A receptor could initiate the signaling cascade that ultimately activates NF-κB (Abdouh et al., 2004; Appert-Collin et al., 2005; Dhandapani et al., 2005; Rojo et al., 2004).

As discussed in the literature review (Chapter II), there are several pro-survival genes that can be transcribed following NF-κB induction, including those that encode for endogenous antioxidant enzymes. In fact, activating NF-κB results in upregulation of three major antioxidant enzymes, Cu/Zn-superoxide (SOD1), Mn-SOD (SOD2), and catalase (Furukawa et al., 1997; Sompol et al., 2006; Zhou et al., 2001b). Evidence from this and other laboratories suggests that such changes might exert neuroprotection. One study suggested that high constitutive NF-κB activity provided neuronal resistance to
hydrogen peroxide-mediated oxidative stress (Lezoualc'h et al., 1998). It is possible that the increased NF-κB resulted in a heightened activation of endogenous antioxidants that provided resistance to oxidative stress.

The studies described in Chapter IV demonstrate that there is early upregulation of SOD1 and SOD2 by ethanol and ipsapirone. Those studies also show that ethanol-induced neuronal apoptosis is prevented by a drug that mimics the effects of SOD and catalase. The current study investigated two potential signaling pathways that might be involved in the early activation of the endogenous antioxidant genes Sod1 and Sod2 by ethanol and/or ipsapirone. The PI-3K and MAPK pathways were the focus of this investigation, because they are reportedly linked to 5-HT1A receptor activation and to downstream activation of NF-κB; NF-κB subsequently augments expression of the genes that encode Sod1 and Sod2.

MATERIALS AND METHODS

*In vitro* fetal rhombencephalic neuronal cultures

The methods used in this study were described previously in several earlier publications from this laboratory (Druse et al., 2005; Druse et al., 2004; Druse et al., 2006; Druse et al., 2007). All animal care and use procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Loyola University Chicago, Stritch School of Medicine. The fetal rhombencephalon, which contains the developing 5-HT neurons (Konig et al., 1988), was removed (Eriksen et al., 2002) from timed-pregnant Sprague-Dawley rats (175-185 grams) at gestation day 14 (G14), where G=0 corresponds to the day of insemination. Throughout the dissection procedure, tissue
samples were kept ice-cold in Hank’s balanced buffered solution (HBSS), and sterile instruments and techniques were used. Rhombencephalic tissue was mechanically disaggregated before seeding onto poly-D-lysine coated plates (Corning, Corning, New York) at a density of $8 \times 10^6$ cells/plate (55 cm$^2$). Cultures were maintained in a neuron-specific chemically modified media (CMM) that included Dulbecco’s Minimal Essential Media/F12 (DMEM/F12) media (Invitrogen, Carlsbad, CA), hydrocortisone-21 sulfate (Sigma, St. Louis, MO), Basal Medium Eagle Vitamin Solution (Sigma, St. Louis, MO), antibacterial agent gentamicin sulfate (Honegger and Monnet-Tschudi, 1997), B27 serum-free medium supplement (Invitrogen, Carlsbad, CA) (Brewer et al., 1993), and 0.25% fetal bovine serum (FBS) (Druse et al., 2004). After 24 hours, 0.4 µM cytosine arabinoside (Sigma, St. Louis, MO) was added to arrest gliogenesis.

Immunohistochemistry was performed using an antibody to glial fibrillary acidic protein (GFAP) to assess the contribution of astrocytes to the cultures; this method confirmed that astrocytes comprised < 5% of the cells in these neuronally-enriched cultures. Cells were grown in control media (no ethanol) for 5 days, with media changes made on alternate days.

Treatments

The fetal rhombencephalic neurons were cultured according to the described method until DIV 5, when 2-hour treatments with ethanol and/or ipsapirone and or enzyme inhibitors were initiated. Using an ethanol chamber system described by this laboratory (Druse et al., 2004), cultures were divided into four groups for RNA isolation and enzyme activity assays. The four treatment groups were Control (no ipsapirone/no
ethanol), Ipsapirone (100 nM ipsapirone), Ethanol (50 mM ethanol), and Ethanol + Ipsapirone (co-treatment with 50 mM ethanol + 100 nM ipsapirone). In addition, either LY294002 (PI-3K inhibitor) or PD98059 (MAPKK inhibitor) were included in samples from each of the treatment groups. The concentration of ipsapirone used in this project promotes the development of 5-HT neurons (Whitaker-Azmitia et al., 1990) and prevents apoptosis in fetal rhombencephalic neurons (Druse et al., 2004).

RNA Isolation and cDNA Synthesis

Trizol reagent (Life Technology, Gaithersburg, MD) (Druse et al., 2006; Druse et al., 2007; Lee et al., 2009) was used to extract total RNA from cultured fetal rhombencephalic neurons. Glycogen (20 mg/ml) (Ambion, Austin, TX) was included to facilitate the precipitation of RNA and to maximize the yield of RNA. RNA was dissolved in 25 µl DEPC-treated H₂O, which was treated with DNA-free (Ambion, Austin, TX) to remove contaminating genomic DNA. RNA was stored at –80° until use. Single strand cDNA was synthesized from 1 to 2 µg of total RNA (DNA-free) using the First Strand cDNA synthesis kit (Fermantas, Hanover, MD).

Quantitative real-time RT-PCR

cDNA (DNA equivalent of 20 ng to 40 ng of total RNA) was diluted with Platinum Quantitative PCR Super Mix-UDG [1.5 U Platinum Taq DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 mM dGTP, 200 mM dATP, 200 mM dCTP, 400 mM dUTP, 1 U UDG (Life Technology)], 0.25 mM Rox (Life Technology), 1/40,000 SYBR Green (Molecular Probes, Eugene, Oregon), and primers
(Druse et al., 2006; Lee et al., 2009). A Perkin-Elmer Gene Amp 7300 Sequence Detector thermal cycler (Applied Biosystems, Foster City, CA) was used for PCR amplifications. RT-PCR data was analyzed using SDS software (Applied Biosystems). Sample inputs were normalized using GAPDH.

Plasmid containing target genes were used to generate standard curves for real-time quantitative RT-PCR assays (Druse et al., 2006), and a standard curve was generated from serial dilutions of known amounts of the input copy number of target genes. Standard curves for GAPDH and genes of interest (10-100,000 copies/µl) were performed in each experiment. Routinely, we included a triplicate RT-PCR reaction lacking cDNA or known DNA template for each set of primers. Specific primary sequences for Sod, Sod2, catalase (Chapter 4) and GAPDH were selected using the Primer Express program (Applied Biosystems) and sequences available from the NCBI database. Primers were synthesized by Life Technology. The forward (F) and reverse (R) primer sequences for rat genes are included in Table 2. Typically, GAPDH and the three target genes were first detected at 22 or 28 cycles, respectively. As previously described (Druse et al., 2006; Druse et al., 2007), data were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical analyses

Data were analyzed using two-way ANOVA and a post-hoc Newman-Keuls procedure to test our hypotheses that LY294002 and PD98059 treatment alters the expression of some NF-κB dependent survival genes in ethanol and/or ipsapirone-treated
neurons to levels found in untreated (control) cultures. A p value < .05 was considered to be significant.

RESULTS

Early upregulation of Sod1 and Sod2 is mediated by the PI-3K and MAPK pathways

Inclusion of the PI-3K inhibitor LY294002 with the treatment groups blocked the ipsapirone-associated increase in Sod1 at 2 hours (p < .01) (Figure 41). The MAPKK inhibitor PD98059 also prevented (p < .01) upregulation of Sod1 at 2 hours (Figure 42). In similar investigations of the Sod2 gene, LY294002 blocked the upregulation of Sod2 found in cultures treated with ethanol, ipsapirone, or ethanol plus ipsapirone Sod2 (p < .01) (Figure 43). Moreover, the MAPKK inhibitor PD98059 prevented the upregulation of Sod2 after a 2-hour treatment of ipsapirone (p < .05), ethanol (p < .01), and co-treatment of ethanol and ipsapirone (p < .05) (Figure 44).

DISCUSSION

The present study determined that early ethanol and/or ipsapirone-induced expression of Sod1 and Sod2, demonstrated in this and the previous chapter, was regulated by PI-3K and MAPK, because inhibitors of these pathways blocked the effects of ethanol and/or ipsapirone. Previously, 5-HT1A receptor stimulation was shown to activate PI-3K and MAPK (Adayev et al., 1999; Druse et al., 2005), augment the effects of NF-κB (Dhandapani et al., 2005; Norris and Baldwin, 1999), and promote survival (Adayev et al., 1999; Hsiung et al., 2005). Also, this laboratory reported that the ipsapirone augments the expression of other NF-κB-dependent pro-survival genes, i.e.,
XIAP and Bcl-xL, and that these changes correlate temporally with neuronal protection against ethanol-induced apoptosis (Druse et al., 2006; Lee et al., 2009).

An interesting finding in this study was that the ethanol-mediated increase in SOD2 expression was inhibited by both LY294002 and PD98059. One mechanism by which ethanol might activate PI-3K and MAPK could involve the rapid rise in ROS. Chapter 3 described the increase in ROS, which begins immediately after ethanol is added to cultures. Consistent with this possibility, Han et al. showed that PI-3K and MAPK are activated by pre-conditioning with a low concentration of hydrogen peroxide; this pre-conditioning also leads to cell survival (Han et al., 2001). Another possible mechanism by which ethanol can activate signaling pathways is through its actions on membrane proteins. In fact, there is evidence that ethanol acts indirectly to activate or inhibit membrane receptors in the CNS. Such receptors include the glutamate receptor and gamma-aminobutyric acid type A (GABA_A) receptor (Lovinger et al., 1989). Ethanol also inhibits the adenosine transporter in rat hippocampus (Diao and Dunwiddie, 1996), thereby increasing the available adenosine to bind to its receptors. Indeed, the A1-adenosine receptor can activate PI-3K (Germack and Dickenson, 2000). Interestingly, a low concentration of ethanol (2 to 20 mM) activates the PI-3K and MAPK pathways, and induces subsequent activation of NF-κB. The same study also suggested that ethanol activates MAPK in PI-3K dependent manner (Liu et al., 2002).

In summary, it appears that the early effects of ethanol and ipsapirone both involve activation of the PI-3K and MAPK pathways. The effects of ipsapirone are likely mediated by the 5-HT1A receptor, which has been linked to both pathways. In contrast, the effects of ethanol are likely to involve the ethanol-associated increase in
ROS and the ROS-sensitivities of both pathways. Whether similar effects of ethanol also occur in vivo are not yet known. It is possible that the low serum concentration used in this study made our neuronal cultures particularly sensitive to oxidative stress (Chan et al., 2001). In addition, although the inhibitor studies strongly suggested that both the PI-3K and MAPK pathways are involved in the early activation of Sod1 and/or Sod2 by ethanol or ipsapirone, the effects of these inhibitors are not 100% selective. Thus, additional studies would help to confirm the specific involvement of these enzymes with the effects of ipsapirone and ethanol.
Figure 41. The early upregulation of Sod1 after 2-hour ipsapirone treatment was inhibited by LY294002, an inhibitor of the PI-3K → pAkt pro-survival pathway. Each value represents the mean ± the SEM of values obtained from 3 to 6 separate experiments. Values are expressed as the fold change in mRNA as calculated by the 2-ΔΔCT method. The results of a 2-WAY ANOVA done on the original (no inhibitor) data are provided in the legends to Figures 19 to 23. A 3-WAY ANOVA (ethanol x ipsapirone x LY) found an interaction of ethanol x ipsapirone x LY [F(1,31) = 18.6, p < .001]. The results of a post-hoc test are depicted by the following notations. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at p < .05 are represented by the *. The # identifies values that are significantly different from the time-matched ipsapirone value at p < .05. The ^ indicates values that are significantly different between treatment versus inhibitor at p < .05.
Figure 42. The early upregulation of Sod1 after a 2-hour ipsapirone treatment was inhibited by PD98059, an inhibitor of the MAPKK → MAPK pro-survival pathway. Each value represents the mean ± the SEM of values obtained from 3 to 6 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. The results of a 2-WAY ANOVA done on the original (no inhibitor) data are provided in the legends to Figures 19 to 23. A 3-WAY ANOVA (ethanol x ipsapirone x PD) found an interactions of ethanol x ipsapirone x PD [$F(1,24)=5.6, p<.05$]. The results of a post-hoc test are depicted by the following notations. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p<.05$ are represented by the *. The # identifies values that are significantly different from the time-matched ipsapirone value at $p<.05$. The ^ indicates values that are significantly different between treatment versus inhibitor at $p<.05$. 
Figure 43. The early upregulation of Sod2 after a 2-hour ipsapirone treatment was inhibited by LY294002, an inhibitor of the PI-3K → pAkt pro-survival pathway. Each value represents the mean ± the SEM of values obtained from 3 to 11 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{ΔΔCT}$ method. The results of a 2-WAY ANOVA done on the original (no inhibitor) data are provided in the legends to Figures 19 to 23. A 3-WAY ANOVA (ethanol x ipsapirone x LY) found a main effect of LY [$F(1,51) = 27.2$, $p < .0001$].

The results of a post-hoc test are depicted by the following notations. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .01$ and $p < .05$ are represented by the * and **, respectively. The ^ and ^^ indicate values that are significantly different between treatment versus inhibitor at $p < .05$ and $p < .05$, respectively.
Figure 44. The early upregulation of Sod2 after a 2-hour ipsapirone treatment was inhibited by PD98059, an inhibitor of the MAPKK → MAPK pro-survival pathway. Each value represents the mean ± the SEM of values obtained from 3 to 11 separate experiments. Values are expressed as the fold change in mRNA as calculated by the $2^{-\Delta\Delta C_T}$ method. The results of a 2-WAY ANOVA done on the original (no inhibitor) data are provided in the legends to Figures 19 to 23. A 3-WAY ANOVA (ethanol x ipsapirone x PD) found a main effect of PD [$F(1,44) = 18.5$, $p < .0001$]. The results of a post-hoc test are depicted by the following notations. Values that are significantly different from the time-matched control (no ethanol, no ipsapirone) value at $p < .01$ and $p < .05$ are represented by the * and **, respectively. The ^ and ^^ indicate values that are significantly different between treatment versus inhibitor at $p < .05$ and $p < .05$, respectively.
Fetal Rhombencephalic Neurons

The rhombencephalon is the primary region of the developing central nervous system that contains the cell bodies that give rise to the 5-HT neurons of the raphe nuclei. Because the rhomencephalon contains a large population of serotonergic neurons, and because ethanol severely damages developing the serotonergic system (Rathbun and Druse, 1985; Sari and Zhou, 2004; Tajuddin and Druse, 1999; Zhou et al., 2001a), utilization of cultures of fetal rhombencephalic neurons provides a means to study possible mechanisms underlying the adverse effects of ethanol on developing serotonin system. Because many of the neurons in these cultures express 5-HT$_{1A}$ receptors (Figure 11), these cultures also provide a means to study the mechanism by which the 5-HT$_{1A}$ agonist exerts its neuroprotective effects.

Ethanol Causes a Rapid Increase and Accumulation of ROS

In the acute ethanol treatment model, it is likely that oxidative stress results from a rapid increase of ROS within the cell. Consistent with findings from the present study, which demonstrated that acute ethanol exposure quickly and progressively increased the generation of ROS in cultures of fetal rhombencephalic neurons, another laboratory also
demonstrated that in vitro acute ethanol treatment resulted in high levels of ROS within 30 minutes in the developing cortical neurons (Ramachandran et al., 2003). Due to difficulties in using the ROS detection dye in ethanol-treated cultures for a prolonged time, it was not feasible to assess ROS levels in cultures treated with ethanol for 24 hours. One complicating factor is the volatility of ethanol in the microplate reader. Another complication is the oxidation of the ROS detection dye even in cultures that lacked ethanol. Future studies of the prolonged and acute effects of ethanol could examine more stable signs of oxidative stress, e.g., levels of oxidized proteins and DNA.

Effects of Ethanol on the Expression of Endogenous Antioxidants

This study showed an early ethanol-associated increase in Sod2 and catalase expression. Studies using inhibitors suggest that the ethanol-induced increase in Sod2 at 2 hours was mediated by the PI-3K and MAPK pro-survival pathways. The early increase in Sod2 and catalase could represent an initial attempt for fetal rhombencephalic neurons to survive. Alone, SOD2, a mitochondrial enzyme, detoxifies superoxide, a precursor to generating more toxic ROS. However, because SOD2 and catalase work in sequence to detoxify mitochondria-damaging superoxide and hydrogen peroxide, respectively (Song and Lee, 2003; Temple et al., 2005; Wheeler et al., 2001), increased expression of the two antioxidants could help keep mitochondrion healthy if this expression was paralleled by changes in enzyme activity. Although the ethanol-associated change in Sod2 was not paralleled by a similar change in enzyme activity at the times measured in our study, the significant increase in catalase was followed by a corresponding elevation in catalase activity. Importantly, investigations from other
laboratories show that an early effect of ethanol was increased expression of SOD and catalase in brain (Enache et al., 2008; Heaton et al., 2003a; Heaton et al., 2003b; Heaton et al., 2003c; Reddy et al., 1999).

Additional studies from this laboratory (Lee et al., 2009) also show that an early response to ethanol produced increased expression of the genes that encode the anti-apoptotic proteins Bcl-2 and XIAP. It is likely that ipsapirone’s ability to block an ethanol-associated decrease in the expression of anti-apoptotic genes or augment such expression is meaningful biologically, because ipsapirone treatment also blocked ethanol-associated apoptosis at 24 hours (Druse et al., 2005; Druse et al., 2004). In a survey of potential ethanol responsive genes, researchers found that even a low concentration of ethanol (50 mM) causes more genes to be upregulated than down-regulated. Among the upregulated genes are neuronal anti-apoptotic genes. The review also suggests that the regulation their expression might involve ROS (Rahman and Miles, 2001).

Any potential protective effects of an ethanol-induced upregulation of antioxidant gene expression are transient. By 24 hours catalase expression in ethanol-treated neurons returned to levels of those in unstressed control neurons. Similarly, Sod1 and Sod2 returned to control levels by 48 hours.

There was also a second ethanol-associated rise in Sod1 and Sod2 at 24 hours. This happened at a time when many cells had already undergone apoptosis. At present, we do not have an explanation for this second rise in the expression of these genes. It is possible that the secondary increase in Sod1 and Sod2 represents another attempt of surviving neurons to resist the damaging effects of ethanol, but it is likely that such an attempt would have been futile because there does not appear to be a corresponding
increase in SOD activity. However, we cannot eliminate the possibility that SOD activity was augmented at a time between 24 and 48 hours that was not examined in this study. Importantly, if these changes were paralleled by changes in enzyme activity, they could actually exacerbate the oxidative stress by overproducing hydrogen peroxide. Oxidative stress could develop, because the ethanol-treated neurons do not have a corresponding increase in catalase expression or activity at that time, and catalase would be necessary to convert hydrogen peroxide to water. In fact, when Sod is overexpressed in fetal hippocampal neurons, there were increased levels of hydrogen peroxide and augmented neurotoxicity (Zemlyak et al., 2006).

Pre-Treatment of Ipsapirone Reduces Ethanol-Mediated ROS Accumulation

Co-treatment with ipsapirone did not prevent the acute ethanol-mediated increases in ROS. These findings strongly suggest that ipsapirone does not as a free radical scavenger. However, when fetal rhombencephalic neurons were pre-treated for 24 hours with ipsapirone, this treatment prevented a rise in ROS when ethanol was added at the end of the pre-treatment period. The time required for ipsapirone to block the ethanol-associated increase in ROS suggests the possibility that ipsapirone’s effects involved the upregulation of one or more genes that encode proteins that prevent an increase in ROS.

Effects of Ipsapirone on the Expression of Endogenous Antioxidants

An intriguing finding in the catalase studies was that there was a main effect of ipsapirone after 8 hours of treatment in our studies of catalase expression. At this early time point, there was an ethanol-induced increase of catalase. The ethanol-mediated
increase in *catalase* was followed by an increased in catalase activity. However, ipsapirone treatment blocked both the rise in expression and activity of this enzyme. Because catalase is both an antioxidant enzyme and the major brain enzymes to metabolize ethanol (Zimatin et al., 2006), it is not clear whether one or both functions were altered by this change. If the increased levels of catalase augmented ethanol metabolism and production of neurotoxic acetaldehyde (Lee et al., 2005) at this early time point, the ipsapirone-mediated blockade of this ethanol effect could help reduce the damaging effects of ethanol. Certainly, these results suggest a possible role of ipsapirone in mediating the regulation of *catalase* expression. However, we must also consider the possibility that ipsapirone’s effects on catalase prevented the cell from detoxifying elevated levels of ROS and led to damaging effects on the cell. As noted, it is essential for the cell to maintain ROS levels in a range that promote its health and to activate NF-κB dependent survival pathways (Lupertz et al., 2008). However, in light of ipsapirone’s neuroprotective effects against ethanol-associated apoptosis, the latter scenario does not appear to be dominant.

At later critical time points, ipsapirone exerted potentially neuroprotective effects on ethanol-treated neurons. Co-treatment with ipsapirone upregulated *catalase* expression at 24 hours and catalase activity at 48 hours. The ipsapirone-mediated elevation of *catalase* at 24 hours and catalase activity at 48 hours could contribute to the neuroprotective effects that are observed when ipsapirone is co-administered with ethanol on a more prolonged basis (> 24 hours). For example, the catalase changes might contribute to ipsapirone-mediated neuroprotection found *in vivo* when ethanol-treated pregnant dams are treated with this or a related drug during the latter portion of gestation.
(Tajuddin and Druse, 1999; Tajuddin and Druse, 2001). These effects could be important to the developing rat brain, where catalase is an important antioxidant to counter the effects of oxidative stress. In fact, the activity of catalase in the developing brain is 550% compared to adult brain (Bayir et al., 2006).

Although the ipsapirone-mediated elevation of catalase activity at 48 hours does not explain ipsapirone’s anti-apoptotic effects at 24 hours, this change could contribute to the neuroprotective effects that are observed when ipsapirone is co-administered with ethanol on a more prolonged basis (> 24 hours). For example, the catalase changes might contribute to ipsapirone-mediated neuroprotection found in vivo when ethanol-treated pregnant dams are treated with this or a related drug during the latter portion of gestation (Tajuddin and Druse, 1999; Tajuddin and Druse, 2001).

It has been shown in many different studies that enhancing the expression of SOD and catalase provide protection against cell death (Dasgupta et al., 2006; Guo et al., 2001; Thiruchelvam et al., 2005; Wheeler et al., 2001). The present study demonstrated that the SOD/catalase mimetic EUK-134 protected developing neurons from ethanol-induced apoptosis. However, when expression of antioxidant enzymes is excessive, as in the over-expression of these genes, it could have an adverse outcome (Brown et al., 1999; Larosche et al., 2009; Zemlyak et al., 2006). Perhaps such adverse effects occur when excessive activity of one antioxidant enzyme could over-produce a reactive intermediate, which would upset the balance in the cell and lead to cell death [reviewed in (Halliwell, 2000)].

In summary, it appears likely that the neuroprotective effects of ipsapirone involve, in part, its ability to modulate the expression of genes encoding antioxidant
enzymes. Certainly, these studies showed that both ethanol and ipsapirone modulate the expression of genes that encode antioxidant enzymes and that some of these changes are paralleled or followed by corresponding alterations in enzyme activity. Although some ipsapirone mediated changes have the potential to lead to an unfavorable balance of ROS or augmented formation of a neurotoxic agent, the evidence that ipsapirone provides neuroprotection against ethanol-induced apoptosis strongly argues against that possibility. Moreover, these studies showed that a mimetic of catalase and SOD exerts neuroprotection against ethanol-associated apoptosis. And finally, pre-treatment of neuronal cultures with ipsapirone attenuates the ethanol-associated rise in ROS. Therefore, these studies provide additional evidence that a 5-HT_{1A} agonist such as ipsapirone might provide protection against fetal alcohol damage to developing 5-HT and other CNS neurons.
Table 3. Summary of Data

<table>
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<tr>
<th>Time</th>
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<th>SOD2</th>
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</tr>
<tr>
<td></td>
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<td>N/C</td>
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<td>N/C</td>
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<td>N/C</td>
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</tr>
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VITA

Jongho Peter Lee was born in Seoul, South Korea to Ik-Jun Lee and Hyung-kung Cho on August 28, 1979. In 1995, Peter began secondary school in the United States at Hoosac High School in upstate New York. Following graduation from Hoosac High School, Peter attended Clark University where he met his wife, Kristen. At Clark, Peter worked as a teaching assistant in the Department of Chemistry and in 2003 received a BA in Chemistry and a BA in Psychology. Peter interrupted his academic studies for a sojourn with the Miz Medi Hospital in Seoul, South Korea. There, Peter began his work with human embryonic stem cell research, which led him to realize his love of biomedical research.

Peter entered the Neuroscience Graduate Program of Loyola University Chicago in August 2003, and joined the laboratory of Dr. Mary J. Druse-Manteuffel. In Dr. Manteuffel’s laboratory Peter started investigating mechanisms that are relevant to Fetal Alcohol Syndrome. Peter was a predoctoral Fellow on the NIAAA T32 Training Grant for the Alcohol Research Program. During his studies, he also took time to attend and present his work at annual meetings including Research Society on Alcoholism (2006, 2007), Society for Neuroscience (2005, 2007, 2008), and International Society for Neurochemistry (2009). He was also actively involved in student affairs of the graduate student council and served as co-president. Peter accepted a post-doctoral fellowship position in the laboratory of Dr. Benjamin Wolozin at Boston University, where he will
study disease processes in neurodegenerative diseases. Peter has been married to Kristen Armandt for seven years. They have one very energetic son, Isaiah.