7-25-2012

Neonatal Isoflurane Exposure on Apoptotic Neurodegeneration and Long-term Behavioral Functions

Susan E. Maloney
University of Missouri-St. Louis

Neonatal Isoflurane Exposure on Apoptotic Neurodegeneration and Long-term Behavioral Functions

Susan E. Maloney

M.A., General Experimental Psychology, University of Missouri – St. Louis, 2007

B.A., Psychology, University of Missouri – St. Louis, 2003

A dissertation submitted to The Graduate School of Arts and Sciences at the University of Missouri – St. Louis in fulfillment of the requirements for the degree Doctor of Philosophy in Psychology with an emphasis in Behavioral Neuroscience

June 2012

Dissertation Committee:

George T. Taylor, Ph.D.
Chairperson

David F. Wozniak, Ph.D.

Michael G. Griffin, Ph.D.

Catherine E. Creeley, Ph.D.

Acknowledgements

I received support and guidance from many people during my journey to complete my doctorate, without which I would not have been able to complete graduate school or the dissertation process. First, I thank Dr. George Taylor, my graduate school advisor, mentor, and the chair of my dissertation committee for his guidance and training over the last decade. Dr. Taylor has been supportive and encouraging since my undergraduate studies. He was an integral part of my decision to pursue a doctorate in Psychology and it was through working in his lab that I discovered a passion for behavioral neuroscience research. His instruction, accompanied by his humor, made my graduate studies so valuable and enjoyable.

I thank Dr. David Wozniak for the opportunity to work in his lab before and during my graduate studies, particularly while I conducted my dissertation study. I greatly value the experience and knowledge I gained through this work and the advice I received, and continue to receive, from Dr. Wozniak. I thank Dr. Michael Griffin for his valued input on my dissertation and throughout the many steps of my graduate studies. I thank Dr. Catherine Creeley for her guidance during the dissertation process, and for sharing her wisdom and laughter throughout my graduate career. Working with Caty is always more fun than work.

I will forever be in debt to Dr. Carla Yuede for her help throughout the dissertation processes. Carla provided me with a great deal of instruction and answered innumerable questions for which I am immensely grateful. Without Carla, I would still be fumbling around the lab trying to figure out what to do.

I thank Dr. John Olney for allowing me to take on this project and for financial support of the study. I am incredibly grateful for the assistance of the members of Dr. Olney's lab, Tracy Wang, Sophie Wang, and Brant Swiney, for their help with and instruction during my histology experiment. To the members of Dr. Wozniak's Animal Behavior Core, Sara Conyers and Josh Dearborn, thank you for putting up with me and for all your help and friendship during my tenure in the Core.

Without the love and support of my amazing family and friends, I would not be who I am today; the person able to follow her dreams and achieve her goals. Thank you for encouraging me every step of the way. Finally, I am extremely grateful to my boyfriend Josh, who is always by my side when I need encouragement and someone to lean on, and who brings an immense amount of love to my life.

Contents

Figures and Tables

Abstract

Clinical studies have reported multiple exposures to anesthesia are associated with an increased risk of later learning disabilities. The general anesthetic Isoflurane (ISO) is commonly used in pediatric anesthesia and decreases the activity of the central nervous system through activation of the $GABA_A$ receptor. Other drug classes, such as anxiolytics, anticonvulsants, and alcohol, that share this mechanism increase neuroapoptosis during the developmental brain growth spurt period. Exposure to these agents during this time also can have detrimental consequences on behavior.

Two experiments were conducted to examine the acute neuroapoptotic response and long-term behavioral impact of postnatal ISO exposure using an *in vivo* mouse model of brain development. In Experiment 1, mouse pups were treated on a single day or across multiple days to 1.5% ISO for 3 hr on postnatal days (PND) 3, 5, or 7. Densities of neurons positively stained for activated caspase-3, an immunohistochemical stain for neuroapoptotic degeneration, were quantified. The greatest neuroapoptotic response was observed in the cortex on PND 5, the caudate on PND 5, the thalamus on PND 3, and the hippocampus on PND 3 and 5. A greater density of cells undergoing apoptosis was observed on PND 5 following a single exposure and on PND 7 following a double exposure.

To examine long-term influence, Experiment 2 followed mice into adulthood after neonatal exposure to isoflurane on PND 3+5+7. Male ISO mice demonstrated increased activity levels as juveniles and slightly heightened anxiety-like behaviors in the EPM as adolescents. During adulthood, female ISO mice failed to exhibit a preference for social novelty by spending comparable time investigating a novel versus a familiar conspecific

during the social approach test. Contextual-cued fear conditioning was also disrupted in female ISO mice as demonstrated by a decrease in freezing behavior.

In conclusion, isoflurane heightened levels of neuroapoptosis, suggesting possible alteration of neural circuitry, and influenced behavior long-term. These findings bring attention to exaggerated cell death during development and modifications in later functioning following developmental exposure to an anesthetic agent. This study provides grounds for developing into adjunctive therapies for the prevention of these disruptions in a clinical setting.

NEONATAL ISOFLURANE EXPOSURE ON APOPTOTIC NEURODEGENERATION AND LONG-TERM BEHAVIORAL FUNCTIONS

Isoflurane (ISO) was created in 1965 and approved for clinical use as a general anesthetic by the Food and Drug Administration in 1981. This agent is non-flammable, has low blood solubility, is highly physically stable, and undergoes little biodegradation. Moreover, ISO does not induce seizures. These qualities make it close to an ideal anesthetic for adults and for children. Despite the development of newer volatile agents, such as sevoflurane and desflurane, ISO remains a standard anesthetic (Ball & Westhorpe, 2007; Eger, 1981).

The class of drugs known as general anesthetic agents depresses central nervous system (CNS) function in a dose-dependent fashion. First the patient experiences sedation then sleep, followed by loss of reflexes, amnesia, unconsciousness, and finally immobility (Julien, 2005). The mechanism for these effects is fairly well known. General anesthetics likely influence the activity of various receptors and channels in the CNS. However, their primary action is enhancing the inhibitory activity of the γ-aminobutyric acid (GABA) type A ($GABA_A$) receptor or decreasing the excitatory activity of the *N*-methyl-D-aspartate (NMDA) glutamatergic receptor (Forman & Chin, 2008). Both the $GABA_A$ receptor and the NMDA receptor are ligand-gated ion channels. The former, when activated by the amino acid neurotransmitter GABA inhibits excitation through the influx of chloride ions (Cl^-) into the neuron. Activation of the NMDA receptor by glutamate does the opposite. Glutamate, also an amino acid neurotransmitter, is excitatory through the influx of sodium ions (Na^+) into the postsynaptic neuron (Forman & Chin, 2008).

Mechanisms of Isoflurane Anesthesia

Isoflurane has multiple sites of action in the CNS. ISO acts at both the pre- and postsynaptic neuron membrane and can potentiate or inhibit functions in its work to depress CNS activity (Van Dort, Baghdoyan, & Lydic, 2008). The primary site of action of ISO is the postsynaptic membrane (Figure 1A; Franks & Lieb, 1994). At the postsynaptic membrane, ISO binds to the $GABA_A$ receptor potentiating Cl^- currents within the neuron, even in the absence of the endogenous GABA neurotransmitter (Jia et al., 2008; Ming, Knapp, Mueller, Breese, & Criswell, 2001). When GABA is present at the receptor, ISO increases the affinity of the neurotransmitter for the $GABA_A$ receptor and prolongs the inhibitory postsynaptic potentials by extending the time the channel remains open. The likely mechanism is a slowing of the release of the GABA neurotransmitter from its receptor (Franks & Lieb, 1994; Van Dort et al., 2008). ISO action at the $GABA_A$ receptor hyperpolarizes the neuron membrane thereby decreasing neuronal activity

ISO also decreases CNS activity through other, secondary mechanisms. ISO activates postsynaptic potassium channels (Figure 1B) and the postsynaptic glycine receptor (Figure 1C). The glycine receptor, similarly to the $GABA_A$ receptor, is a ligand-gated Cl^- channel. ISO action at these sites decreases neuronal excitability just like ISO potentiation of $GABA_A$ receptors (Kopp Lugli, Yost, & Kindler, 2009). While the glycine neurotransmitter has its own receptor channel, glycine also has a binding site on the NMDA receptor. *In vitro* studies suggest ISO inhibits NMDA function by competitively binding to the glycine site on the NMDA receptor (Figure 1D; Dickinson et al., 2007; Van Dort et al., 2008). ISO also has been suggested to decrease glutamatergic activity by

influencing glutamate reuptake at the presynaptic membrane (Figure 1E; Kopp Lugli et al., 2009; Van Dort et al., 2008). Few *in vivo* studies using rodent models have been able to confirm these ISO effects on the glutamatergic system (Van Dort et al., 2008).

Additional secondary mechanisms of ISO occur at the presynaptic neuron. ISO depresses CNS activity through actions at the presynaptic neuron by directly influencing neuronal firing. ISO impedes neurotransmitter release by preventing synaptic vesicle exocytosis (Hemmings, Yan, Westphalen, & Ryan, 2005; Herring, Xie, Marks, & Fox, 2009). This is accomplished through the inhibition of the voltage-gated Na^+ channels that potentiate the action potential to the presynaptic terminal, independent of postsynaptic $GABA_A$ potentiation (Figure 1F; Herring et al 2009; Lingamaneni, Birch, & Hemmings, 2001; Ouyang, Herold, & Hemmings, 2009; Shiraishi & Harris, 2004). Some researchers have suggested that the influx of Ca^{2+} in the nerve terminal also may be impeded (Wu, Sun, Evers, Crowder, & Wu, 2004). The different actions of ISO occur in different areas of the CNS. Although ISO influences many pre- and postsynaptic receptors and channels, ISO action is most potent at the postsynaptic $GABA_A$ receptor (Ming et al., 2001).

The anesthetic phenotypic outcome of ISO results from action in various areas of the CNS. Sedation is from a general slowing of brain activity (Kopp Lugli et al., 2009; Rau et al., 2009). Loss of consciousness is likely a result of disruptions in the neural circuits involving the cerebral cortex, thalamus and reticular activating system (Kopp Lugli et al., 2009; Vanini, Watson, Lydic, & Baghdoyan, 2008). Amnesia may result from effects on the hippocampus, amygdala, prefrontal cortex, and the sensory and motor cortices (Kopp Lugli et al., 2009). ISO action at glycine receptors in the spinal cord contributes to inhibition of spinal reflexes and startle responses by blocking the spinal

NMDA receptor (Franks & Lieb, 1994; Hemmings, 2009; Kopp Lugli et al., 2009; Stabernack et al., 2003).

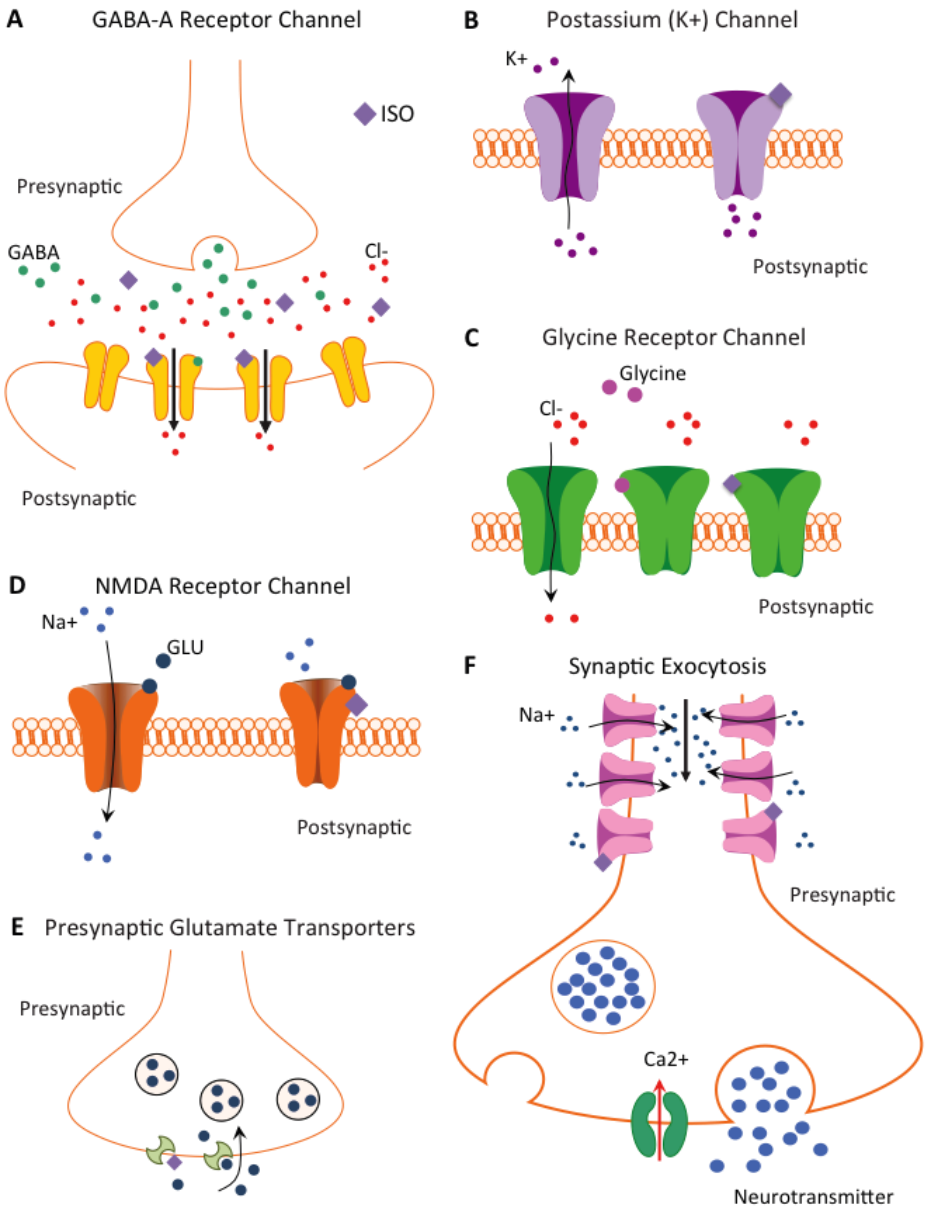

Figure 1. ISO has multiple sites of action in the CNS, at both the pre- and postsynaptic neuron membrane, in its work to depress CNS activity. (A) The primary mechanism of ISO is to bind to and enhance the function of the postsynaptic $GABA_A$ receptor, thereby inducing an influx of Cl^- into the neuron. ISO also has many secondary actions, including (B) activating the postsynaptic potassium channels and (C) activating the postsynaptic glycine receptor. ISO has been suggested to inhibit the activity of the glutamatergic system by (D) binding to the glycine site on the postsynaptic NMDA receptor and blocking the influx of Na^+ thereby inhibiting the depolarization of the neuron, and (E) influencing the reuptake of glutamate at the presynaptic neuron membrane. (F) ISO also impedes neurotransmitter release by preventing synaptic vesicle exocytosis by blocking the voltage-gated Na^+ channels that potentiate the action potential to the presynaptic terminal.

Animal Models of Adult Isoflurane Exposure

Because ISO acts on the major neurotransmitter systems in the brain, any negative effects of ISO exposure need to be understood. Rodent models provide researchers with the means of empirically investigating ISO influence on neurons and behavior.

To the healthy rodent brain, ISO exposure is not particularly destructive, but does trigger some neurons to commit suicide. However, the research is equivocal due to the different analytic techniques used in different studies. Studies have demonstrated no damaging influence of ISO exposure by using an analytic technique that quantifies general neurodegeneration. The brains of adult rats exposed to 2.4% ISO for 4 hr did not display any increase in neurodegeneration compared to room air-exposed control rats with the FluoroJade staining (Stratmann et al., 2010; Stratmann, Sall, et al., 2009). FluoroJade-B staining detects neurodegeneration that arises from a variety of mechanisms, including, but not selective for, neuroapoptosis (Schmued, Albertson, & Slikker, 1997). On the other hand, other studies demonstrate neuronal death following ISO exposure using a different analytic technique that is more specific to apoptotic neurodegeneration, or neuroapoptosis, a form of natural programmed neuron death. Activated caspase-3 (AC3) staining specifically evaluates neuroapoptosis. Caspase-3 activation is an important step in neuroapoptosis (D'Mello, Kuan, Flavell, & Rakic, 2000). It occurs at the beginning of the neuron death process and takes place throughout the entire neuron. Because of this, AC3 staining is a very useful method in marking neurons undergoing apoptosis (Olney, Tenkova, Dikranian, Muglia, et al., 2002; Olney, Tenkova, Dikranian, Qin, et al., 2002). AC3 staining 6 hr following 1.2% or 1.4% ISO exposure for 2 hr revealed a neuroapoptotic response to the ISO in both the rat and mouse

brain (Lin & Zuo, 2011; Xie et al., 2008). Conversely, increased neuroapoptosis was not demonstrated by AC3 staining in young adult mouse brains following 0.6%, 1.3%, or 2% ISO exposure for 3 hr. The 2% ISO exposure did slightly reduce cell proliferation and cause a delayed reduction of astroglial processes in the hippocampus and dentate gyrus (Dallasen, Bowman, & Xu, 2011). The results from these studies suggest ISO is not toxic enough within the healthy adult rodent brain to reliably wipe out large groups of neurons, but ISO is toxic enough to induce otherwise healthy neurons to undergo apoptosis and die.

Studies of the behavioral consequences of ISO exposure in adult rodents are mainly focused on cognitive functioning, particularly spatial memory functioning. The Morris water maze (MWM), the radial arm maze (RAM), and the Barnes maze are widely used spatial memory tasks in rodent research. Performance in these tasks is considered to highly rely on the functioning of the hippocampus and its networks (Sharma, Rakoczy, & Brown-Borg, 2010).

ISO exposure does not affect spatial memory of adult mice and rats in the same manner. Acute spatial memory performance in the mouse is impaired after repeated, long-duration exposures to ISO. Quick recovery of function often occurs. Mice exposed to 1% ISO for 2 hr for 5 consecutive days exhibited disrupted spatial reference memory in the MWM 4 days later (Bianchi et al., 2008). Exposure to 1.4% ISO for 2 hr for 5 consecutive days disrupted spatial reference memory acquisition in the MWM in mice 2 days after exposure, but not two weeks after exposure (Su, Zhao, Wang, Li, et al., 2011).

The spatial memory performance of mice is not disrupted following a single exposure to ISO. Repeated exposures to a short duration of ISO also do not cause acute

spatial memory deficits in mice. The MWM performance of mice was not impaired 1 day after a single 2 hr or 6 hr exposure to 1.4% ISO (Su, Zhao, Wang, Li, et al., 2011). A single exposure or repeated exposures to 1.4% ISO for 30 min did not influence performance of mice in the Barnes maze one day after last exposure (Butterfield, Graf, Ries, & MacLeod, 2004).

Spatial memory performance of the rat is more sensitive to disruption by ISO exposure than that of the mouse. Unlike mice, rats exhibit longer disruptions of spatial reference memory performance after a single long-duration exposure to ISO. However, performance deficits do not extend to spatial working memory. Impaired spatial reference memory performance of rats in the Barnes maze was observed 2 weeks after a single exposure to 1.2% ISO for 2 hr (Lin & Zuo, 2011). In contrast, a single exposure to a similar concentration of ISO, 1.8% ISO for 2 hr, did not influence performance of adult rats in a working memory RAM task two weeks after exposure (Crosby, Culley, Baxter, Yukhananov, & Crosby, 2005).

Spatial acquisition impairments appear in adult rats following a low concentration and duration of ISO, but, interestingly, no acquisition impairments have been observed following higher concentrations at longer durations. Learning deficits in the MWM were observed in young adult rats following 1% ISO for 1 hr. Interestingly, deficits were not observed in rats exposed to a higher ISO concentration (1.5% or 2%) for 1 hr (Valentim et al., 2010). No learning deficits were observed in the MWM following 1.3% ISO for 4 hr in young or middle-aged adult rats. Similar to other studies, both ages groups exhibited retention memory deficits (Callaway, Jones, & Royse, 2012).

Another memory test considered heavily to involve the hippocampus and its networks by many researchers is the conditioned fear test of associative learning. While the contextual-dependent portion of this test is thought to primarily involve hippocampal function, the time gap between exposure and testing portions relies heavily on prefrontal cortical activity (Bryan, Lee, Perry, Smith, & Casadesus, 2009). Rats exposed to a single exposure of 1.2% ISO for 2 hr displayed impaired performance during the hippocampal-dependent portion of conditioned fear task 2 weeks after exposure (Lin & Zuo, 2011). These findings indicate exposure to ISO has a complex influence on the cognitive systems of the rodent brain.

This idea of the complex influence of ISO on the rodent brain continues with a report of improved cognitive functioning following ISO exposure. A single exposure to 1.3% ISO for 2 hr improved the performance of adult mice 1 day after exposure in a modified holeboard task assessing visuospatial abilities (Rammes et al., 2009). One week later, however, this improvement disappeared.

Aged rodent cognition may be more susceptible to ISO-induced disruptions than young adult rodent cognition. The spatial memory performance of young adult mice declines after repeated exposures to longer durations of ISO. In contrast, spatial memory performance of aged mice declines after a single exposure to a long duration of ISO. Spatial memory acquisition in the MWM was impaired in aged mice 2 weeks after exposure to 1.2% ISO for 6 hours compared to controls (Su, Zhao, Wang, Xu, et al., 2011). Similar to that in young adult mice, short durations of ISO exposure, one or multiple, do not have a negative impact on the spatial memory performance of aged mice. Aged mice exposed to 1.4% ISO for 30 min for one or multiple episodes displayed no

cognitive impairment when tested in the Barnes maze the next day (Butterfield et al., 2004). Longer durations, i.e. 2 hours or more, of ISO exposure appear to be a key in provoking spatial reference memory impairments in rodents.

Even though spatial memory performance deficits occur in young adult rats two weeks following a longer duration of ISO exposure, aged rats exposed to a single longer duration of ISO do not display spatial memory deficits when the time between exposure and testing is expanded greatly. Aged rats exposed to 2.1% ISO for 4 hr performed similar to controls when tested in the MWM 4 months later (Stratmann et al., 2010). The discrepancies in these findings do not suggest that aged rats are not spatially impaired by ISO exposure like younger rats. The differences in time between exposure and testing do not allow for that comparison. Data is needed on aged rat function 2 weeks after ISO exposure. However, we can assume that long-term spatial impairment is unlikely in aged rodents exposed to a single episode of ISO. Very similar discrepancies exist in conditioned fear results. Younger rats exhibit fear-conditioning deficits two weeks after ISO exposure. Aged rats demonstrated no fear conditioning impairments 4 months after exposure to 2.1% ISO for 4 hr (Stratmann et al., 2010). These findings do not support the clinical findings that age is a risk factor for the development of postoperative cognitive dysfunction in elderly patients (Moller et al., 1998). However, multiple exposures to ISO may be necessary, and ISO itself may not be the factor influencing human cognition.

Rat working memory function becomes more sensitive to ISO influence with age. Unlike young adult rats, aged rats exhibit impaired spatial working memory function when exposed to the same ISO exposure and testing conditions as the younger rats. Aged rats exposed to 1.2% ISO for 2 hr performed worse in a working memory RAM task

relative to room air exposed controls when cognitive testing occurred two weeks following ISO exposure (Culley, Baxter, Crosby, Yukhananov, & Crosby, 2004).

The findings of adult rodent ISO exposure studies suggest learning and memory performance that relies heavily on the function of the hippocampus and its networks is susceptible to disruption from ISO, especially the performance of the rat. However, ISO influences the rodent brain in a complex manner that likely affects many areas and neuronal networks involved in cognition.

While studies of the influence of ISO exposure on healthy adult or aged rodent models remains somewhat unclear, research with Alzheimer's disease (AD) transgenic mouse models indicates a negative impact of ISO on AD pathology. Increases in AD neuropathology resulting from ISO exposure have been demonstrated in healthy adult rodents as well as AD transgenic mice. Healthy mice exposed to ISO exhibit increased beta-site amyloid precursor protein-cleaving enzyme and amyloid-beta (A beta) protein demonstrated by Western blots analysis and immunoblot detection (Xie et al., 2008). The Tg2576 transgenic mouse is used as an animal model for studying AD pathology because this mouse shows age-dependent AD neuropathology, specifically elevated levels of brain amyloid at 1 year (Perucho et al., 2010). Another AD mouse model, the JNPL3 Tau transgenic mouse, develops severe neurofibrillary tangles by 1 year of age (Planel et al., 2009). An increase in respective AD pathology in both transgenic mouse models occurs following ISO exposure (Perucho et al., 2010; Planel et al., 2009). *In vivo* studies suggest, regardless of duration of exposure, higher concentrations of ISO potentiate A beta-induced apoptosis, while lower concentrations of ISO attenuate A beta-induced apoptosis (Perucho et al., 2012; Xu et al., 2011).

While ISO may potentiate AD pathology in mice, its capability to potentiate related behavioral declines runs into "floor effects" in the performance of AD transgenic mice. Behavioral deficits exhibited by AD transgenic mice reach a point at which no further declines are possible even with the addition of other adverse factors. The Tg3576 transgenic mouse exhibits a decrease in ambulations compared to wild-type controls in the Y-maze spontaneous alternation task. This was unchanged by ISO exposure. However, ISO exposure did significantly reduce the exploratory behavior in this task (Perucho et al., 2010). Many areas of the brain, i.e. hippocampus, septum, basal forebrain, and prefrontal cortex, contribute to working memory performance in the Y-maze spontaneous alternation task. In the same mouse model, MWM performance was impaired in the transgenic mice compared to wild-type controls prior to ISO exposure. Following ISO exposure, performance of the wild type (WT) mice declined in the MWM, while that of the transgenic mice did not (Bianchi et al., 2008). These studies demonstrate that while AD pathology impairs cognitive functioning and ISO exposure can further increase that pathology, the direct impact of ISO exposure on declining cognition may be minor or difficult to document because of "floor effects" in performance. Anesthetic exposure adversely affects some areas of cognition in AD transgenic mouse brain, yet the impact of the AD pathology on hippocampal-involved spatial memory appears to supersede any further damage by ISO.

Disturbances in function in WT aged rats following ISO exposure may not be dependent upon AD pathology outside of the transgenic models. Non-transgenic aged rats exposed to 1.4% ISO for 2 hr demonstrated deficits in MWM acquisition, however, these deficits did not appear to be A beta- or tau-neuropathogenesis-dependent (Liu et al.,

2012). It is possible some other ISO-induced mechanism is responsible for the behavioral declines in these aged rats.

The rodent model research does not suggest ISO is neurotoxic to the adult rodent brain, even though ISO may induce an apoptotic response. ISO acutely disrupts some aspects of cognition in the adult rodent, but these disruptions do not appear to be long lasting, even in aged rodents. ISO does have a negative impact on the brains and behavior of AD transgenic mice. However, the AD pathology is so disruptive to cognition that exposure to ISO does not worsen performance. The influence ISO on the adult rodent brain is complex. Indeed, as discussed below, ISO can even be protective during certain types of brain injury.

Animal Models of Isoflurane Neuroprotection

In certain circumstances, ISO exposure can have a neuroprotective influence in the rodent brain. This occurs mainly following insults from hypoxia-ischemia (HI) or traumatic brain injury (TBI). ISO has been shown to provide neuroprotection from ISO-induced neurotoxicity within *in vitro* pre-conditioning paradigms. Pre-conditioning rat cortical neurons with smaller or equal concentrations of ISO 24 hr before ISO exposure inhibited ISO-induced neurotoxicity (Wei, Liang, & Yang, 2007). Whether this neuroprotection occurs *in vivo* and has any impact on subsequent behavior is not known. However, ISO protection against brain damage and behavioral disturbances has been observed in *in vivo* rodent models.

ISO can reduce damage to brain tissue caused by an HI event. Pre-, peri-, and post-conditioning with ISO reduces infarct volumes in rat brains following unilateral HI. Infarct volumes were reduced in rats exposed to 1.5% or 2.25% ISO for 1 hr for 5

consecutive days or 30 min prior to HI insult and with 2% ISO for 1 hr after HI insult (Lee, Li, Jung, & Zuo, 2008; Li & Zuo, 2009; Xiong et al., 2003). ISO preconditioning also decreased the neurological deficits observed in these rats (Xiong et al., 2003). ISO (1.8% for h hr) exposure concomitant with the unilateral HI event also resulted in decreased infarct volume 7 hr, 1 day or 4 days later and an acute reduction in apoptosis (Kawaguchi et al., 2004). Reductions in infarct volumes and apoptosis are not long lasting. ISO exposure (1.5 % or 2% for 1 or 2 hr) decreased brain edema and attenuated cell death at 24 hr but not 72 hr, as well as improved neurobehavioral deficits in mice with subarachnoid hemorrhage (Altay et al., 2012; Khatibi et al., 2011)

While experimental research demonstrates ISO provides acute, not chronic, protection against HI insult in rat brain tissue, limited research exists evaluating this influence on retention of behavioral function. *In vivo* behavioral studies have investigated the neuroprotective effect of ISO in response to HI insult and TBI. Motor coordination performance was improved in rats with unilateral HI insult on an accelerating rotarod test with pre- and post-conditioning with 1.1% and 2.2% or 2% ISO, respectively (Lee et al., 2008; Li & Zuo, 2009). These improvements lasted short-term (24 hr) and long-term (4 weeks). The presence of ISO during a unilateral HI event may improve cognitive performance in rats. The presence of 1.4% ISO during 10 min of near complete forebrain ischemia enhanced the performance of the ischemic rats to that of sham-operated rats in spatial learning MWM trials.(Elsersy et al., 2004). However, rats exposed to 70% nitrous oxide-fentanyl also performed similarly to sham operated rats in this study, thus, making it difficult to infer about the ability of ISO to protect spatial learning functions. ISO exposure during a hypoxic event protected against declines in a spatial working memory

in the mouse. Although hypoxic mice exhibited only a transient deficit in performance in the Y-maze, the addition of 1.2% ISO during 1 hr of hypoxia removed this deficit such that the ISO-exposed hypoxic mice performed comparably with controls (Bekker, Shah, Quartermain, Li, & Blanck, 2006).

ISO may a very mild protective effect on TBI. Pre- and post-conditioning with 1% ISO for 30 min improves beam walking and MWM performance in rats with TBI. However, this improvement is relative to rats pre- and post-conditioned with fentanyl, a narcotic analgesic, not room-air exposed or sham-operated controls (Statler et al., 2006). Thus, it is hard to know if the ISO improves performance or if the fentanyl disrupts performance. Deep ISO exposure (>2%) for 2 hr has been shown to increase neurodegeneration and worsen neurobehavioral outcomes at 4 hr and 48 hr in rat model of TBI (Hertle et al., 2012). ISO exposure may provide some benefit to TBI, but only at very low concentrations and durations.

ISO neuroprotection in the neonatal rodent is similar to that in the adult rodent, in that neuroprotection is acute, not chronic. However, histological outcomes appear to be species-dependent and, perhaps, age-dependent. Pre-conditioning with 30 min of 1.5% ISO exposure prior to HI brain injury on postnatal day (PND) 6 or PND 7 in the rat has resulted in decreased neuronal loss in the cortex and hippocampus, and an increase in overall brain weight (Zhao, Peng, Li, Xu, & Zuo, 2007; Zhao & Zuo, 2004). Rat pups pre-conditioned with 2% ISO for up to 21 min on PND 7 or 10 exhibited reduced infarct volumes (Chen et al., 2011). Short-term (7 days) protection against neuronal loss in the hippocampus occurred when PND 7 rat pups were pre-conditioned with 2% ISO for 1 hr immediately before the rat pups were subjected to unilateral HI (Sasaoka et al., 2009).

This protection did not extend out to 49 days. Benefits from ISO pre-conditioning do not occur in the mouse brain after neonatal unilateral HI. ISO pre-conditioning (1.8% for 2-3 hr) in PND 9 mouse pups did not reduce short-term or long-term neuronal loss in the hippocampus, retrosplenial cortex, or subiculum (McAuliffe, Joseph, & Vorhees, 2007; McAuliffe et al., 2009). Perhaps only shorter, not longer, durations of ISO exposure are beneficial to the neonatal brain. Furthermore, neonatal preconditioning with ISO may have different outcomes in the different species.

Neuroprotection against behavioral insults following neonatal unilateral HI presents differently than in the adult rodent. Juvenile motor coordination was improved in HI rats with 1.5% ISO pre-conditioning for 30 min or 2.1% ISO for 21 min as demonstrated by improved performance on a rotarod task (Chen et al., 2011; Zhao et al., 2007). Striatal motor control is preserved by 1.8 % ISO exposure for 3 hours prior to HI in PND 9 mouse pups as demonstrated in the apomorphine challenge (McAuliffe et al., 2007). Improvement in performance during this task also occurred when tested as adults (McAuliffe et al., 2007). No improvement in long-term sensorimotor abilities occurred with 2% ISO pre-conditioning for 1 hr in PND 7 rat pups when tested as adolescents (Sasaoka et al., 2009).

ISO has a very mild influence on cognitive functioning in unilateral HI rodent models. Adolescent mouse non-spatial memory function in the novel object recognition task was preserved by 1.8% ISO for 2 hr (McAuliffe et al., 2007) although 1.8% ISO preconditioning for 3 hr had no impact on spatial memory function in mice when tested during adolescence or adulthood (McAuliffe et al., 2007; McAuliffe et al., 2009).

Protection from HI-induced behavioral disruption by ISO includes motor coordination whether induced in the adult or neonatal rodent. The neonatal brain appears less responsive to ISO protection of other behavioral domains than the adult brain. Protection does not extend to sensorimotor abilities or hippocampal-involved spatial memory in the neonatal rodent model as in the adult rodent model. However, non-spatial memory may be preserved in the neonatal rodent.

Although experimental data supports a neuroprotective role of ISO during certain types of brain tissue injury in the rodent, evidence does not exist of any long-term neuroprotective effects. Limited rodent research, however, demonstrates short-term and long-term improvements in behavioral function.

Vulnerability of the Perinatal Rodent Brain

The CNS is vulnerable to insult by exogenous substances during each stage of development. The result of the insult is partially dependent on the timing of the insult. For instance, craniofacial deformities occur following fetal alcohol exposure during the first trimester (West, 1987). Excessive alcohol exposure during the second trimester disrupts neurogenesis resulting in decreased numbers of neurons and decreased brain weight. Alcohol exposure also can have detrimental effects on function without external indicators. Alcohol exposure during the final stage of brain development, which occurs in human infants from the third trimester of gestation to 2 or 3 years of age, can disrupt cognitive functioning and decrease brain weight without decreasing body weight (Dobbing & Sands, 1979; West, 1987). Rapid growth occurs during this period; therefore, it is commonly referred to as the "brain growth spurt" period. This growth results from the many developmental processes taking place during this final period of brain

maturation, including cell migration, neuronal differentiation, dendritic growth and gliogenesis. Perhaps most importantly, significant levels of synaptogenesis, or the creation of synapses, are also occurring during the brain growth spurt period. Disruption of the formation of synaptic connections can have devastating consequences on brain functioning.

The brain growth spurt period is a sensitive period for disruption of brain development from exposure to environmental influences. This period occurs in rats and mice perinatally from immediately before birth until 2 weeks of age (Dobbing & Sands, 1979; West, 1987). The result is that neonatal rodents are ideal for investigating drug influences on this period of brain development because manipulations can be done after birth.

It is important to match stages of brain development when translating developmental rodent research to the appropriate human age. As stated above, the brain growth spurt period occurs in rodents in the first 2 postnatal weeks whereas this stage of brain development lasts in humans from the third trimester of gestation until 2 to 3 years of age (Dobbing & Sands, 1979; West, 1987). Weaning of mice and rats occurs circa PND 21, and they remain in the prepubertal, or juvenile, stage until approximately PND 39. In rodents, the pubertal rise of gonadotropins and resulting sex steroids begins around 40 days of age (Cohen, Dore, Robaire, & Ruf, 1984; Diaz Rodriguez et al., 1999). The adolescent designation is then reserved for the peri- and post-pubertal animals from 40-59 days of age. By PND 60 rodents are considered young adults, while physiological evidence suggests full maturity is reached at PND 100 or later (Robb, Amann, & Killian, 1978).

Many different categories of drugs have a negative impact on the developing brain during the brain growth spurt period, which can have negative consequences on later behavior. These include both drugs of abuse, such as alcohol and phencyclidine (PCP), and drugs developed for therapeutic purposes, such as anxiolytics, anesthetics, and anticonvulsants (Fredriksson, Archer, Alm, Gordh, & Eriksson, 2004; Satomoto et al., 2009; Stefovska et al., 2008; Wozniak et al., 2004; Young et al., 2005; Yuede et al., 2010). These drug classes share one of two mechanisms in the CNS: NMDA-antagonism or $GABA_A$-agonism.

Animal models of neurodevelopment demonstrate the harmful influence NMDA-antagonistic and $GABA_A$-agonistic agents in the developing brain. Substances that influence other neurotransmitter systems in the brain, such as the cholinergic or dopaminergic systems, do not have the same detrimental effects during development as NMDA-antagonists or GABAmimetics (Ikonomidou et al., 1999). It appears that only manipulations of the glutamatergic and GABAergic systems during the brain growth spurt period directly result in acute neuronal damage and long-term behavioral consequences.

These two classes of drugs, $GABA_A$ agonists and NMDA antagonists, damage the developing CNS through the same process, although the mechanism of action of each drug class is different. Drugs acting at $GABA_A$ and NMDA receptor sites increase apoptosis, or programmed cell death, when administered during the brain growth spurt period (Dikranian et al., 2001). Apoptosis is an inherent neuron deletion mechanism that controls neuron populations (Kerr, Wyllie, & Currie, 1972). In the developing brain, the process of apoptosis deletes unusable or flawed neurons. Olney and colleagues defined a

second type of neuron death, excitotoxicity. Excitotoxicity is a form of neuron death that results from over-stimulation of the neuron cell membrane by glutamate (Olney, 1974). It is now well understood that these are two separate and ultrastructurally different forms of neuron death (Ishimaru et al., 1999).

Rodent models of the adverse effects of NMDA antagonism. Research in the last decade and a half demonstrates an abnormal increase in neuroapoptosis during the brain growth spurt period can result in long-term functional deficits. Drug-induced increases in developmental neuroapoptosis were first observed in the postnatal rat brain following exposure to the NMDA antagonists dizocilpine (MK801), PCP, ketamine, and carboxypiperazin-4-yl-propyl-1-phosphonic acid (CPP). The increased apoptotic neurodegenerative response was observed between the developmental ages of PND 0 and 14 with peak sensitivity from PND 3 to 7 (Ikonomidou et al., 1999). Subsequent research corroborated the increased neuroapoptosis with ketamine exposure in the postnatal rodent brain (Scallet et al., 2004; Straiko et al., 2009; Young et al., 2005; Zou et al., 2009). An increase in apoptotic neurodegeneration following ketamine exposure has also been demonstrated in the developing nonhuman primate brain (Slikker et al., 2007; Wang et al., 2006).

Further research confirmed that the acute damage caused by NMDA antagonists could influence later behavior. Exposure to MK801 (3 x 0.5 mg/kg) or ketamine (50 mg/kg) outside the period of peak neuroapoptotic sensitivity (PND 10 and PND 11, respectively) increases neurodegeneration in mouse pups relative to saline vehicle-treated controls. Behavioral testing early in adulthood revealed altered locomotor activity levels in the treated mice. Treated mice demonstrated an initial period of hypoactivity followed

by hyperactivity for the remainder of a 60 min testing session (Fredriksson & Archer, 2003, 2004; Fredriksson et al., 2004). This indicates an inability of the NMDA antagonist-treated mice to habituate to the testing environment.

This inability of the treated mice to habituate to the testing environment suggests a possible deficiency in cognitive functioning. Further investigation of possible cognitive deficits were conducted using the RAM and circular swim path tasks, which assess spatial reference memory and depends on the functioning of networks involving the hippocampus. The treated mice failed to demonstrate improvement across time in the RAM task. The treated mice, also, were unable to locate a new platform location in the circular swim path task following 3 days of acquisition. (Fredriksson & Archer, 2004; Fredriksson et al., 2004). Given the results of the activity testing session, it is possible that the inability of the animals to demonstrate correct learning of new information was due to hyperactivity within the apparatus, not actual cognitive deficits. Although, an impaired learning capacity is evident in NMDA-treated mice, including an inability to switch focus within a task.

The findings of a subsequent study indicate NMDA antagonist-induced learning impairments are independent of altered locomotor activity. Again, mice were treated with an NMDA antagonist on PND 10 and behaviorally tested in early adulthood. A reduced dose of ketamine (25 mg/kg from 50 mg/kg) produced the initial hypoactivity during the 60 min activity testing session, similar to mice treated with 50 mg/kg ketamine, but no hyperactivity during the remainder of the testing session. The ketamine-exposed mice again failed to show improvements over time in the RAM task (Fredriksson, Ponten, Gordh, & Eriksson, 2007). The mice treated with the reduced dose of ketamine continued

to exhibit impaired RAM performance without demonstrating hyperactivity. This suggests that the learning deficits observed in the RAM and circular swim path task are due to impaired learning capacity in NMDA antagonist-treated mice, not to hyperactivity within the apparatus.

It is important to note here that the reduced ketamine dose increased neurodegeneration relative to controls; however, this effect was not statistically significant. It is possible that cognitive behaviors are more sensitive to postnatal NMDA antagonism than are general locomotor activity deficits. In addition, deficits in behavior may not be necessarily caused only by increased neuronal death, but perhaps also by disruption of other important developmental processes occurring at that time.

The cognitive consequences of other NMDA-antagonist dosing regimens have been investigated. Multiple exposures to an NMDA antagonist at different ages during the brain growth spurt period produce robust learning deficits beyond that observed with a single exposure. Mice exposed to PCP on PND 2 (35 mg/kg) or PND 7 (50 mg/kg) produced an increase in neuroapoptosis (Yuede et al., 2010). When tested as juveniles, and then again in early adulthood, the mice treated with PCP on PND 2 or PND 7 demonstrated mild cognitive deficits. Exposure to PCP on both PND 2 and 7 not only produced an increase in neuroapoptosis beyond that observed in mice exposed only on PND 7, but also largely affected cognitive performance. Robust and long-term cognitive impairments were observed in the MWM and the conditioned fear paradigm, indicating deficits in both spatial and non-spatial learning and memory function, respectively, in mice exposed to PCP on both PND 2 and 7. Specifically, these mice demonstrated impaired acquisition and retention at the juvenile stage (PND 30), early adulthood (PND

75) and later adulthood (PND 170). Exposure to an NMDA antagonist at multiple ages during the brain growth spurt period appears to have an additive or synergistic influence on neuroapoptotic damage and behavioral disturbances.

Chronic exposure to a NMDA antagonist at ages during the brain growth spurt period later than PND 7 also impairs later learning in the rodent. Rats chronically exposed to MK801 (0.2 mg/kg or 0.4 mg/kg) or the competitive NMDA antagonist CGS 19755 (2 mg/kg or 4 mg/kg) from PND 7 – 20 failed to reach criterion during RAM testing in early adulthood (Kawabe, Iwasaki, & Ichitani, 2007). These findings, along with Yuede et al. (2010), reveal NMDA-antagonist exposure during the brain growth spurt period acutely damages the developing neurons and disrupts later cognitive function.

Significantly lower body weights were observed in the treated animals compared to control animals in both Yuede et al. (2010) and Kawabe et al. (2007). This occurrence was most likely due to the incapacitating state produced by the treatment, which prevented feeding on multiple days. It is possible the deficits observed are an indirect result of malnutrition. However, in Yuede et al. (2010) differences in weight gains were only observed until PND 9, suggesting the impairment was caused by the direct influence of the NMDA antagonist on the developing CNS.

Rodent studies have exposed the harmful consequences of postnatal exposure to NMDA antagonists on adult cognitive function, as well as the many factors influencing this impairment, including dose level, age of exposure, and number of exposures.

Rodent models of the adverse effects of $GABA_A$ agonism. Following the discovery that NMDA antagonism induces an increase in apoptotic neurodegeneration in

the developing brain, Ikonomidou, Bittigau, et al. (2000) investigated agents that potentiate or antagonize receptors of other major neurotransmitter systems. Only GABA-mimetic agents, specifically those that act to potentiate the $GABA_A$ receptor, produce the increased neuroapoptotic response induced by NMDA antagonists. Many drugs that potentiate the GABA-ergic system are used for therapeutic purposes, such as anticonvulsants and general anesthetics. Despite the beneficial aspects of these drugs, their activation of the $GABA_A$ receptor makes their use risky in the developing brain. Both anticonvulsant drugs (phenobarbital, pentobarbital, diazepam, clonazepam, and vigavatrin) and general anesthetic drugs (propofol, sevoflurane, and ISO) increase neuroapoptosis in the neonatal rat brain when administered during peak brain growth spurt period (Bittigau et al., 2002; Cattano, Young, Straiko, & Olney, 2008; Ikonomidou, Bittigau, et al., 2000; Satomoto et al., 2009).

Although the harmful consequences of neonatal exposure to anticonvulsants have been known for decades (Holmes et al., 2001; Jones, Lacro, Johnson, & Adams, 1989; Speidel & Meadow, 1972), little was understood about the mechanism for neurotoxicity. The finding that anticonvulsant drugs induce abnormal neuroapoptosis through the activation of $GABA_A$ receptors provides a potential mechanism. Yet, not all research supports this possibility. Despite demonstrating increased neurodegeneration in the laterodorsal thalamus following diazepam treatment (5 mg/kg) on PND 10 in mouse pups, no changes in activity levels or RAM performance were observed in adulthood (Fredriksson et al., 2004). However, PND 10 may not be a very sensitive age for increased neurodegeneration following acute anticonvulsant exposure.

Whether or not an increased neuroapoptotic response is observed following GABAmimetic exposure can depend on the histological methodologies employed. No neuroapoptotic response was observed in rat pups following phenobarbital (50 mg/kg) treatment on PND 6. However, the lack of a neuroapoptotic response is likely due to the fact that the rats were sacrificed 24 hr after initial drug exposure and most evidence of apoptosis has likely undergone phagocytosis by that time (Kerr et al., 1972). Nevertheless, reductions in newly born cells were revealed. Phenobarbital decreased cell proliferation in several areas of the rat brain, including the neocortex, caudate, thalamus, dentate gyrus, and corpus callosum, when administered every other day for 5 consecutive days starting on PND 0, 3, 6, or 14, totaling three treatments, 50, 40, and 30 mg/kg, respectively. Phenobarbital treatment on PND 6, 8 and 10 also suppressed neurogenesis in the dentate gyrus on PND 15 (Stefovska et al., 2008).

Learning deficits were revealed in rats treated with phenobarbital chronically during the entire brain growth spurt period. In a hippocampus-involved spatial learning version of the water maze, rats treated with phenobarbital (50 mg/kg) daily between PND 0 – 14 exhibited impaired acquisition when tested on PND 180 (Stefovska et al., 2008). Despite not demonstrating an enhanced neuroapoptotic response, this study correlates neurodevelopmental disruptions with later cognitive decrements following anticonvulsant treatment.

Rodent studies have employed chronic postnatal exposures to anticonvulsants to investigate the behavioral consequences of a dosing regimen more closely related to a clinical dosing regimen. Phenobarbital treatment (40 or 50 mg/kg) on PND 2 – 21 reduced working memory performance in mice in the RAM during adulthood, which

requires proper hippocampus and prefrontal cortex functioning (Yanai et al., 1989). Rats treated with diazepam (10 mg/kg) at the same ages (PND 2 – 21) exhibited mildly impaired locomotor coordination, but no impairments in the RAM during very early adulthood (Schroeder, Humbert, Desor, & Nehlig, 1997). Chronic anticonvulsant treatment during development can impair cognition, especially working memory, although these impairments may be dose- and age at testing-dependent in the mouse.

Chronic postnatal anticonvulsant treatment also modifies aggressive behaviors. Chronic daily treatment on PND 1 – 21 with diazepam (1, 5, or 10 mg/kg), lorazepam (0.25, 0.5, 1, or 2.5 mg/kg), or clonazepam (1 – 5 mg/kg) in rat pups altered behaviors in the resident intruder paradigm when tested in early adulthood (File, 1986a, 1986b, 1986c). Aggressive behaviors towards an intruder in the home cage and submissive behaviors as the intruder of a conspecific's home cage increased in the treated rats. However, the higher dosages also increased aggressive behaviors towards the conspecific resident when tested in the conspecific's home cage. Rats chronically treated with diazepam (10 mg/kg) during only the early brain growth spurt period (PND 1 – 7) exhibited lower exploratory behavior and locomotor activity in the holeboard, increased aggressive behaviors towards an intruder, and decreased anxiety when tested as juveniles (File, 1987). Chronic postnatal treatment with $GABA_A$-potentiating anticonvulsant drugs produces hyperaggressive behaviors in the rodent. This may result from changes to serotonergic system function produced directly or indirectly by the drug exposure, given the serotonergic system has been suggested as underlying differences in aggressive behaviors (Miczek, Maxson, Fish, & Faccidomo, 2001).

Many general anesthetics potentiate the $GABA_A$ receptor, such as the intravenous agents thiopental, propofol and etomidate, and the inhalational agents ISO, sevoflurane, and desflurane (Dickinson, de Sousa, Lieb, & Franks, 2002; Irifune et al., 2003; Jia et al., 2008; Ming et al., 2001; Nishikawa & Harrison, 2003; Tomlin, Jenkins, Lieb, & Franks, 1998). Similar to the results of studies involving the GABA-mimetic anticonvulsants, rodent research has demonstrated the potential for general anesthetics to influence behavior following developmental exposure. Rats, administered propofol (3 x 30 mg/kg) on PND 6 displayed impaired non-spatial exploratory learning in a holeboard apparatus when tested on PND 49. Unlike control rats, propofol-treated rats exhibited no decrease in nose pokes or distance traveled and an increase in number of rears in the holeboard from day 1 to day 2, indicating a lack of habituation to the apparatus (Bercker et al., 2009). Spatial learning and memory performance in the MWM task was not impaired in rats treated with propofol. A second cohort of rats in the same study was exposed to 3 – 5% sevoflurane for 6 hr on PND 6. Sevoflurane exposure did not influence habituation to the holeboard or MWM performance (Bercker et al., 2009). Furthermore, a different study exposed mice to the same concentration of sevoflurane (3% for 6 hr) and found no difference in these mice from controls in general exploratory behavior or spatial working memory performance in the Y-maze alternation task (Satomoto et al., 2009).

However, non-spatial learning impairment in the sevoflurane-treated mice was reported in this study. Sevoflurane-treated mice displayed hippocampal- and amygdala-involved non-spatial learning impairments in the conditioned fear paradigm during adolescence (PND 56) and adulthood (PND 100 – 120). A separate study demonstrated impaired freezing behaviors in mice in the contextual and auditory cue fear conditioning

tests during adolescence following 1 MAC sevoflurane and desflurane for 3 hr (3% and 8%, respectively) on PND 6 (Kodama et al., 2011). Non-spatial learning and memory function is perhaps more sensitive to postnatal general anesthesia exposure at these concentrations than the spatial cognitive functioning required by the MWM.

Deficits in social memory and social interaction have also been observed. Mice exposed to 3% sevoflurane for 6 hr on PND 6 demonstrated impaired memory for a female conspecific relative to control mice at PND 130 (Satomoto et al., 2009). This suggests the adult mice exposed to sevoflurane during the brain growth spurt period had impaired social memory. Additionally, the sevoflurane-exposed mice failed to demonstrate proper social interaction by spending the same duration of time investigating a conspecific as an inanimate object, whereas control mice spent more time investigating the conspecific. Differently, exposure to sevoflurane (3%) or desflurane (8%) for 3 hr on PND 6 did not alter behaviors in the EPM during adolescence, suggesting postnatal anesthesia exposure at these levels does not influence on anxiety levels at this age (Kodama et al., 2011).

Exogenously induced potentiation of the $GABA_A$ receptor during the brain growth spurt period of brain development has negative influences on later cognitive, social and emotional behavior. This likely is due, at least partly, to an increase in neuroapoptosis induced by these GABA-ergic agents.

Rodent models of the adverse effects of combined NMDA antagonism and $GABA_A$ agonism. Animal research into the influence of ethanol (EtOH) on the developing brain during the brain growth spurt period was initiated following Ikonomidou and colleagues' (Ikonomidou et al., 1999) initial discovery of the

neuroapoptotic response produced by developmental NMDA antagonist exposure. EtOH is of interest in the anesthetic literature because it is an NMDA antagonist and a $GABA_A$ agonist, and all anesthetics fall into one of these two categories (Harris et al., 1995; Hoffman, Rabe, Moses, & Tabakoff, 1989; Lovinger, White, & Weight, 1989).

Suspicion of a link between maternal EtOH intake and offspring abnormalities is not new, yet it was not until 1970s that the disruption of fetal development by maternal EtOH intake became a clinically diagnosable condition, fetal alcohol syndrome (FAS; Jones & Smith, 1975). Fetal EtOH-induced brain abnormalities can be present in the absence of any external criteria used to define FAS. Of particular importance is that the most vulnerable period for disruption of brain development from EtOH exposure is the brain growth spurt period (Clarren, Alvord, Sumi, Streissguth, & Smith, 1978; West, 1987).

Rodent research confirmed postnatal exposure to EtOH triggers an increase in neuroapoptosis during the brain growth spurt period in both rats (Dikranian, Qin, Labruyere, Nemmers, & Olney, 2005; Ikonomidou, Stefovska, & Turski, 2000) and mice (Dikranian et al., 2005; Olney, Tenkova, Dikranian, Qin, et al., 2002; Wozniak et al., 2004; Young & Olney, 2006) as evaluated by AC3 staining and through electron microscopic observation of ultrastructural criteria. The neuroapoptotic response in neonates is more widespread with EtOH than that induced by other NMDA antagonists or GABA agonists, suggesting that it is the combined drug effect that is most damaging (Ikonomidou, Bittigau, et al., 2000).

Exposure to drugs that target only the GABA system such as the benzodiazepines (diazepam and clonazepam) and barbiturates (pentobarbital and phenobarbital) induce

widespread apoptotic neurodegeneration in a pattern that is different from that triggered by NMDA antagonists. Indeed, a composite of the damage from an NMDA antagonist and a $GABA_A$ agonist closely resembles the pattern of damage observed with EtOH (Ikonomidou, Bittigau, et al., 2000). Studies have subsequently demonstrated the enhancement of neuroapoptosis induced from the combined administration of an NMDA antagonistic agent and a separate $GABA_A$ agonistic agent beyond that observed with the administration of either agent alone (Fredriksson et al., 2004; Fredriksson et al., 2007; Young et al., 2005).

EtOH exposure during the brain growth spurt period can reduce brain mass without reducing overall body growth (West, 1987). Ikonomidou, Bittigau, et al. (2000) suggest this reduced brain mass can be explained by the dramatic cell deletion that occurs through increased apoptosis. This cell loss is also suggested to be responsible for the detrimental effect of EtOH exposure during the early postnatal period on later behavior. An undesirable influence on later behavior by the enhanced neuroapoptotic response would mean the brain does not recover from this acute damage during development.

To a certain extent, this appears to be the case. Severe deficits in learning and memory function later in life have been observed in a mouse model of neonatal EtOH exposure that also demonstrated a widespread EtOH-induced neuroapoptotic response. Mice exposed to EtOH (2 x 2.5 g/kg) on PND 7 exhibited impaired acquisition and memory retention performance on MWM task as juveniles (PND 30) compared to saline treated control mice (Wozniak et al., 2004). Some recovery of function was demonstrated when these mice were re-tested as adults (PND 150 – 180), although there was still evidence for learning and memory impairments at this age. This indicates postnatal EtOH

exposure disrupts spatial learning and memory at an early age, but that some functional recovery is possible.

To clarify if this functional recovery was simply a product of aging, occurring in the absence of any previous exposure, a separate cohort of mice was exposed to EtOH (2 x 2.5 g/kg) on PND 7, but initial MWM testing was not conducted until early adulthood (Wozniak et al., 2004). When initially tested on the MWM as adults (~PND 75), EtOH-exposed mice exhibited impaired acquisition but not impaired retention memory. Again, these mice were re-tested later in adulthood (PND 240). This re-testing yielded acquisition deficits, albeit smaller, and still no impairment of retention memory in the EtOH-exposed mice. The findings from this second cohort of mice indicate functional recovery from the juvenile stage to adulthood is probably due to pretraining at such an early age, as the mice initially tested in adulthood were still impaired. Over time, the brain must recover or adapt in some way following the increased neurodegeneration, as the second cohort of mice did not exhibit the retention memory impairments the first cohort exhibited. However, complete functional recovery is not likely.

The second cohort of mice in this study was also tested in the RAM to examine spatial working memory performance (Wozniak et al., 2004). RAM testing was conducted well into adulthood on approximately PND 150. The EtOH-treated mice demonstrated mildly impaired spatial working memory acquisition performance relative to control mice. The mice were not assessed in the RAM as juveniles, as with the MWM, due to the food restriction requirements of the task. As a result, it is not known whether greater working memory impairment would have been observed at that age. Regardless, this study demonstrates postnatal EtOH treatment compromises hippocampus-involved

reference and working memory in the rodent, which is most likely due to the NMDA-antagonistic and GABA-mimetic properties of EtOH.

Further evidence of impaired function has been demonstrated with neonatal exposure to separate GABA-mimetic and NMDA-antagonist agents. Disruption of adult cognitive function was demonstrated with the combination of ketamine and thiopental or propofol (Fredriksson et al., 2007). Mice were administered a combination of 25 mg/kg ketamine plus 5 mg/kg thiopental or 10 mg/kg propofol on PND 10 and tested in the RAM during late adolescence to early adulthood, between PND 55 – 70. Treated mice failed to improve over time in the RAM task relative to vehicle-treated controls and mice receiving monotherapy, demonstrating the greater functional damage was produced by the combination of both agents over either alone.

Rodent research clearly warns of the danger of the combination of NMDA antagonism and $GABA_A$ potentiation on the developing CNS and behavioral functions. Learning and memory function are especially at risk, during both the juvenile stage and adulthood.

Isoflurane in the Neonatal Rodent Brain

General anesthetic agents primarily act to either block NMDA receptor activity or enhance $GABA_A$ receptor activity. For the most part, in the healthy adult brain, this action in the CNS does not have a detrimental effect. However, as discussed above, NMDA receptor inhibition and $GABA_A$ receptor potentiation can adversely influence the developing brain during the brain growth spurt period. Early damage caused by these agents can translate into behavioral deficits later in life.

ISO primarily acts to potentiate the $GABA_A$ receptor, but as described above, it has also been suggested that ISO acts at many other locations in the CNS, although with less efficacy. As ISO is used in clinical practice for pediatric anesthesia (Lonnquist, 2002), it is important to elucidate any harmful effects ISO may have on the developing brain given its primary mechanisms of action in the CNS.

Histological evidence of isoflurane neurotoxicity. Current surgical anesthesia protocols employ a combination of NMDA-antagonists and $GABA_A$ agonists to induce and maintain general anesthesia. Rodent research provides histological and behavioral evidence of the harm this current protocol can have in the developing brain. Seven-day-old rats were exposed to nitrous oxide (N_2O), midazolam, or ISO only or in all combinations (Jevtovic-Todorovic et al., 2003). Acute histopathology revealed the combination of all the three agents (9 mg/kg i.p. midazolam, 0.75 vol% ISO for 6 hr, and 75 vol% N_2O for 6 hr) induced a robust increase in neurodegeneration in many brain areas of PND 7 rats compared to all other treatment groups. There were no increases in apoptotic neurodegeneration relative to control rats in the rats that received N_2O only for 6 hr or midazolam only. However, ISO only (0.75. 1.0 or 1.5 vol%) for 6 hr increased apoptotic neurodegeneration in a dose dependent fashion relative to room air exposed controls 8 hr after the start of exposure. The laterodorsal and anteroventral thalamic nuclei showed the greatest damage, even at the lowest ISO exposure. The parietal cortex, by contrast, showed significantly greater damage compared to controls only at the highest ISO exposure. That ISO is the only agent increasing apoptotic responses on its own suggests this agent is the most influential. Indeed, enhanced neuroapoptosis in the

hippocampus and cortex has been confirmed in PND 7 rats following 0.75% ISO for 6 hr (Jiang et al., 2012)

The dose-dependent response of the PND 7 rat brain to ISO exposure has been indicated by other studies. Exposure to a higher concentration (3.5%) of ISO for only 4 hr in the PND 7 rat brain exhibited decreased progenitor proliferation in the dentate gyrus and increased neurodegeneration in the thalamus, hippocampus, cortex, and mammillothalamic tract relative to control conditions (Stratmann, May, et al., 2009; Stratmann, Sall, et al., 2009). A lower concentration of ISO (0.55%), however, for 2, 4, 6, or 8 hr did not increase apoptotic neurodegeneration in these brain regions in the PND 7 rat brain 8 hr after cessation of treatment (Zou et al., 2008).

Subsequent studies have confirmed the developing rat brain is most sensitive to the neurotoxic effects of ISO during the brain growth spurt period. Investigation into the effect of ISO exposure in the rat at different ages (PND 1, 3, 10 and 14) revealed 1.5% ISO for 6 hr on PND 1 or PND 3 increased apoptosis neurodegeneration in the thalamus and cortex whereas no differences were observed on PND 10 or PND 14 between any ISO concentration and room-air exposed control rats (Yon, Daniel-Johnson, Carter, & Jevtovic-Todorovic, 2005). Thalamic damage was greater when ISO exposure occurred on PND 3 compared to exposure on PND 1.

An increase in neuroapoptosis occurs following ISO exposure at even the earliest age of the brain growth spurt period in the rat, gestational day 21 (G21). Rat fetuses exposed to 3% ISO for 1 hr on G21 exhibited increased apoptotic response in the CA1 region of the hippocampus and the retrosplenial cortex 6 hr after exposure (Wang et al., 2009). A lower concentration of ISO (1.3% for 1 hr) did not induce these

neurodegenerative differences. Another study found this lower concentration for a longer duration (6 hr) on G21 actually decreased apoptosis in the CA1 region of the hippocampus and in the retrosplenial cortex 8 hr after initial exposure (Li et al., 2007). This suggests a higher concentration of ISO is required to induce neuroapoptosis at the earliest time of the brain growth spurt period in the hippocampus and retrosplenial cortex.

ISO exposure outside of the brain growth spurt period does not have the same neurotoxic influence. Rats exposed to ISO (1.5% for 30, 60, or 120 min) outside of the sensitive brain growth spurt period (PND 16) did not show any evidence of increased neuronal death within the prefrontal cortex (Briner et al., 2010). Exposure to ISO (1.7%) for 35 min on 4 consecutive days starting on PND 14, extending past the brain growth spurt period, resulted in a decrease in neuronal density in the granule cell layer of the dentate gyrus in adulthood but not during the juvenile period, and decreased neurogenesis in the dentate gyrus when assessed immediately following the final ISO exposure. However, no evidence of increased neuroapoptosis was found in the dentate gyrus or CA1 region of the hippocampus 24 hr after last ISO exposure (Zhu et al., 2010). This is not surprising given that 24 hr after exposure most evidence for apoptosis will be gone (Kerr et al., 1972). Also, these rats only received one exposure to ISO during the sensitive brain growth spurt period, PND 14, an age past the peak of development for neurons in the hippocampus (West, 1987). The decreased neurogenesis in the dentate gyrus was still present in ISO-exposed rats 4 weeks following exposure, indicating a long-lasting influence on neurogenesis.

Based on these studies in the rat, the hippocampus is most sensitive to ISO-induced neuroapoptosis earlier in the brain growth spurt period, i.e. PND 7 and earlier.

The thalamus is most vulnerable between PND 3 and 7. The cortex, however, appears to be sensitive to the neuroapoptotic-inducing influence of ISO throughout the brain growth spurt period, especially to high ISO concentrations.

The neurotoxic influence of ISO is not limited to the developing rat brain. A dose-response study conducted in C57BL6 mice demonstrated increased neuroapoptosis is dependent upon concentration and duration of ISO exposure. Five to seven-day-old mice were exposed to ISO at 0.75% for 4 hr, 1.5% for 2 hr, or 2% for 1 hr and the apoptotic response in the brain was evaluated 5 hr after initial exposure (Johnson, Young, & Olney, 2008). Compared to the litter-matched control groups, the ISO exposure increased apoptosis in the striatum by 477%, 178%, and 325%, respectively, indicating that ISO induces a robust neurotoxic effect in the mouse brain and that the response is greatest in the striatum with a low concentration of ISO for a long duration. Furthermore, this study reveals neither duration nor concentration to be the rate-limiting factor for damage. A low concentration for a longer duration may produce a comparable response as a high concentration for a shorter duration.

ISO-induced neurodegeneration has been demonstrated in other regions of the developing mouse brain regardless of ISO concentration with longer duration of exposure. Six-hr exposures to ISO at both a lower concentration (0.75%) and a higher concentration (2.7%) induced apoptotic neurodegeneration in the PND 7 mouse hippocampus and cortex, respectively (Istaphanous et al., 2011; Liang et al., 2010). Mice exposed to 1.5% ISO for 6 hr on PND 7 exhibited increased neurodegeneration in the cerebral cortex, thalamus, and hippocampus, with evidence that at least the cortical neuron death was apoptotic in nature, when assessed 8 hr following initial exposure

(Loepke et al., 2009). However, no differences in neuronal densities were observed between anesthesia-exposed mice and controls in the cortex or hippocampus in adulthood (~PND 84). A slightly higher ISO concentration for a shorter duration (2% for 3 hr) on PND 6 increased neuroapoptosis in the mouse cortex and dorsal hippocampus (Kodama et al., 2011).

Rodent histology reveals the striatum, cortex, thalamus, and hippocampus are at the greatest vulnerability to ISO-induced neuroapoptosis between PND 3 – 7. An increase in neuroapoptosis, however, does not always denote changes in adult neuronal densities. More importantly, evidence exists that this early response translates into a behavioral phenotype.

Functional consequences of developmental isoflurane exposure. The PND 7 rats exposed to the triple anesthetic cocktail, N_2O (75 vol% for 6 hr), midazolam (9 mg/kg), and ISO (0.75 vol% for 6 hr), exhibited diminished cognitive abilities when tested as juveniles compared to control rats, specifically impaired spatial memory acquisition in the MWM (Jevtovic-Todorovic et al., 2003). Retesting during adulthood revealed impaired spatial reference memory retention in the MWM and impaired spatial working memory acquisition in the RAM. While this study indicates an insult t cognitive function following neonatal exposure to combined GABA-mimetic and NMDA-antagonist anesthesia, it did not investigate behavioral consequences of the ISO alone-induced apoptotic response. Subsequent studies have furthered the investigation into ISO-induced behavioral outcomes.

The behavioral impairments observed following neonatal ISO exposure in the rat predominately are cognitively based. Rats exposed to ISO (1 – 2%) briefly (10 min) on

the day of birth exhibited impaired spatial learning in during the MWM as adolescents and impaired spatial reference memory during RAM testing as young adults (Rothstein, Simkins, & Nunez, 2008). Exposure to a higher concentration (3.5%) of ISO for 4 hr on PND 7 did not influence juvenile MWM performance or conditioned fear outcome (Stratmann, Sall, et al., 2009). When these ISO-exposed rats were re-tested in adulthood in the MWM, acquisition impairments were not evident, but impaired retention memory performance was exhibited. A separate cohort of rats in the same study was treated with ISO in the same manner but not behaviorally tested until adulthood. Impaired spatial acquisition in the MWM was observed in these rats compared to control rats. The ISO-exposed rats exhibited impaired responses in the hippocampal-dependent contextual portion of the conditioned fear test, but not the amygdala-dependent auditory tone portion of the test. A related study also found the same ISO exposure impaired MWM spatial reference memory performance in young adult rats, but did not influence MWM working memory performance or conditioned fear responses (Stratmann, May, et al., 2009). ISO exposure during the early ages of the brain growth spurt period may induce both spatial and non-spatial cognitive functional deficits, which do not appear until after the juvenile period.

Studies of ISO exposure during late, and even after, the developmental brain growth spurt period suggest cognitive functional deficits may be observed earlier when exposure occurs at a later age. Object recognition performance, a non-spatial memory task, was impaired in the ISO-exposed rats during the juvenile stage and was even more pronounced in adulthood, indicating the functional deficit worsened over time (Zhu et al., 2010). Rats were exposed to 1.7% ISO for 35 min daily from PND 14 to PND 17. This

suggests the early rodent brain may recover in such a way that behavioral disturbances do not present until after puberty. This would indicate the disturbances are dependent upon hormone activation.

Spatial memory deficits following ISO-exposure during development are not as pronounced in the mouse as in the rat. No differences were observed in the MWM in young adult mice exposed to 1.5 % ISO for 6 hr on PND 7 compared to controls (Loepke et al., 2009). However, this MWM protocol did not include cued trials used to teach the mice the how to function in the pool, i.e. to find the platform, prior to spatial memory acquisition trials. It is hard to know if this influenced the spatial memory acquisition and retention memory performance. A lower concentration of ISO (0.75%) for the same 6 hr duration on PND 7 also did not influence juvenile MWM performance in the mouse (Liang et al., 2010). This study did employ cued trials to ensure no non-cognitive performance differences and that all mice could properly perform the task. Mice exposed to ISO (1.7% for 35 min) on PND 14 for 4 consecutive days did not exhibit impaired spatial learning in a novel test designed to assess place learning when tested as adolescents (Zhu et al., 2010). However, the mice demonstrated impaired reversal learning. It is hard to understand exactly what this impairment indicates since this is a novel spatial learning task. Despite that, impaired reversal learning performance could indicate an inability to discriminate between tasks and limited cognitive flexibility in the ISO-treated mice. Impairments have been observed in the conditioned fear paradigm during adolescence following postnatal ISO exposure. Mice were exposed to 2% ISO for 3 hr on PND 6 and fear conditioning was tested on PND 49. The ISO-exposed mice displayed decreased freezing behavior in both the hippocampal-involved contextual

conditioning test and the amygdala-involved auditory-cue conditioning test (Kodama et al., 2011).

The mice data suggest that ISO disrupts cognition, but not MWM performance as in the rat data. The MWM paradigm may not be sensitive enough to demonstrate the cognitive deficits induced by ISO in the mouse, as it is in the rat. The conditioned fear data clearly suggest, however, the presence of cognitive disruptions.

Investigations into other behavioral domains are limited. Mice exposed to ISO on PND 7 (1.5 % ISO for 6 hr) displayed no differences in locomotor activity over a 60 min testing session compared to control mice (Loepke et al., 2009). Emotional behavior findings are equivocal. Rats exposed to ISO (1 – 2%) briefly (10 min) on the day of birth spent significantly less time in the open arms of the elevated plus maze (EPM) and made significantly fewer percentage of entries into the inner regions of an open field chamber compared to control rats when tested late in adolescence (Rothstein et al., 2008). However, rats exposed to ISO *in utero* made more entries into the open arms of the EPM compared to control rats with no differences between time spent in the open arms when tested during late adolescence (Palanisamy et al., 2011).

Overall, ISO exposure during the earlier ages of developmental brain growth spurt period (PND 7 and earlier) likely impairs cognitive function later in life, although the research is inconsistent. It is probable that some cognitive disruption is occurring, but it is dependent upon exposure concentration, duration, and age of exposure and testing paradigm employed. It is also uncertain whether other behavior domains are adversely affected by this exposure, such as social and emotional functioning, as these domains have not been investigated as extensively as learning and memory.

Supporting Clinical Evidence

Clinical evidence provides ample justification for conducting a comprehensive evaluation of anesthetic drugs in the developing brain. Wilder et al. (2009) studied the association between anesthetic exposures before 4 years of age and the development of reading, written language, and math learning disabilities (LD). This study found that a single exposure of anesthesia was not associated with an increased risk of LD but children who received two or three anesthetic exposures were at an increased risk of LD, and that the risk for LD increased with longer cumulative duration of anesthetic exposure. A matched cohort study found multiple, but not single, anesthesia exposures before the age of 2 increased the risk of developing LDs (Flick et al., 2011). A retrospective cohort analysis of children who underwent hernia repair surgery during the first 3 years of life found these children were more than twice as likely to be diagnosed with a developmental or behavioral disorder later in life (DiMaggio, Sun, Kakavouli, Byrne, & Li, 2009). The assessment of the neurobehavioral development of children who underwent urologic surgery before or after the age of 2 found children who underwent surgery prior to the age of 2 demonstrated more behavioral disturbances than those who underwent surgery after the age of 2, although the results were not statistically significant (Kalkman et al., 2009). The results of these studies demonstrate the importance of pre-clinical research into any possible adverse neurobehavioral consequences of anesthesia exposure. While surgery is unavoidable in many situations, an understanding of possible anesthesia-induced insult will help clinicians uncover the best protection against such insult.

Focus of Current Study

The focus of the current study was to clarify the patterns of neuroapoptosis induced by postnatal ISO exposure, and specifically that caused by single versus multiple ISO exposures across different ages of the brain growth spurt period. Moreover, this study sought to illuminate the behavioral consequences of multiple postnatal ISO exposures in the neuromotor, cognitive, and socio-emotional domains.

EXPERIMENT 1

Research with rodent models of CNS development reveals that a certain level of neural apoptosis is normal in the developing brain. However, exogenous agents enhancing the activity of the $GABA_A$ receptor may induce neuroapoptosis beyond normal developmental levels (Bittigau et al., 2002; Cattano et al., 2008; Ikonomidou, Bittigau, et al., 2000; Satomoto et al., 2009). The general anesthetic ISO is one such agent. ISO induces an abnormal increase in neuroapoptosis when administered to mice or rats during the brain growth spurt period of development (Jevtovic-Todorovic et al., 2003; Johnson et al., 2008; Loepke et al., 2009). It is important to understand any harmful influence ISO exposure may have on the developing brain since ISO is commonly used in pediatric anesthesia (Lonnquist, 2002).

The purpose of the first experiment was the investigation of possible different patterns of abnormal neuroapoptosis depending on developmental age at and number of ISO exposures. Mouse pups were exposed to the anesthetic at one of three different ages or at multiple ages during the brain growth spurt period. Densities of neurons positively stained for AC3, an immunohistochemical stain for neuroapoptotic degeneration, were quantified in the caudate, cerebral cortex, dorsal hippocampus, thalamus, and all areas

combined (total brain area). The primary hypothesis was that a single postnatal ISO exposure would produce an enhanced density of AC3-positive profiles in the total brain area and separately in all brain regions examined (cerebral cortex, caudate, dorsal hippocampus, and thalamus) compared to control animals exposed to room air. Secondarily, the degree of the abnormal increase in density of AC3-positive profiles was hypothesized to depend on the age at which exposure occurs given the ontogenetic stage at which the different brain structures mature (West, 1987). That is, the greatest increase in density of AC3-positive neurons in the thalamus and hippocampus would occur on PND 3, in the caudate on PND 5, and in the cerebral cortex on PND 7. Thirdly, it was hypothesized that the pattern of the abnormal increase in density of AC3-positive neurons would be altered with multiple postnatal ISO exposures versus a single postnatal ISO exposure.

Methods and Materials

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri – St. Louis and the Washington University Animal Studies Committee. The 83 experimentally naïve C57BL6 mouse pups were from 15 different litters. All mice were maintained in the animal colony in the Department of Psychiatry at the Washington University School of Medicine. This mouse strain was chosen because it is often used for knockout or transgenic animal models, and the results from this study will serve as a foundation for future studies involving genetic modification. The colony room lighting was a 12:12 hr light/dark cycle; room temperature (~20-22°C) and relative humidity (50%) was controlled automatically.

Standard lab diet and water was freely available. Pregnant dams were individually housed in translucent plastic cages measuring 28.5 cm x 17.5 cm x 12 cm with corncob bedding.

Experimental Design

The first experiment was designed to investigate the influence of ISO on neuroapoptosis in the postnatal mouse brain at different ages and with different numbers of exposures. The mice were exposed to ISO or room air on six different postnatal ages: PND 3; PND 5; PND 7; PND 3+5; PND 5+7; or PND 3+5+7. Experimental groups were as follows:

Group 1: PND 3 ISO exposed (n = 7)
Group 2: PND 3 litter-matched room-air exposed controls (n = 6)
Group 3: PND 5 ISO exposed (n = 7)
Group 4: PND 5 litter-matched room-air exposed controls (n = 6)
Group 5: PND 7 ISO exposed (n = 10)
Group 6: PND 7 litter-matched room-air exposed controls (n = 6)
Group 7: PND 3+5 ISO exposed (n = 7)
Group 8: PND 3+5 litter-matched room-air exposed controls (n = 6)
Group 9: PND 5+7 ISO exposed (n = 8)
Group 10: PND 5+7 litter-matched room-air exposed controls (n = 6)
Group 11: PND 3+5+7 ISO exposed (n = 8)
Group 12: PND 3+5+7 litter-matched room-air exposed controls (n = 6)

Procedures

Anesthesia exposure. At the appropriate ages, PND 3, 5, 7, 3+5, 5+7, or 3+5+7, pups in the experimental groups from each litter were exposed to the general anesthetic isoflurane USP (Butler Animal Health Supply, Dublin, OH) at a concentration of 1.5 % for 3 hr in an anesthesia flow chamber as previously described (Johnson et al., 2008). Age- and litter-matched pups were exposed to room air for 3 hr to serve as controls. The concentration and duration of ISO exposure was chosen based on previous pilot studies demonstrating 1.5% for 3 hr induced an apoptotic response without high mortality or

excessive weight loss, and because it is below the minimal alveolar concentration (MAC) for the infant mouse. The MAC is the concentration of anesthesia at which 50% of subjects do not move in response to a noxious stimulus. The peak MAC for ISO is 1.87% in infants humans 1 to 6 months of age (Cote, Lerman, Ward, Lugo, & Goudsouzian, 2009) while the peak MAC for the infant mouse is 2.26% (Loepke, McCann, Kurth, & McAuliffe, 2006). Therefore, the concentration of 1.5% ISO is below the MAC for both the human infant and the infant mouse. A sub-MAC, and therefore sub-clinical, concentration of ISO was ideal for this study. Should subclinical concentrations produce histological or behavioral deficits, one would expect to see similar, if not greater, deficits with higher concentrations.

Pups were weighed prior to treatment on each exposure day and placed in a flow chamber inside a temperature-regulated water bath, which maintained a constant temperature of 34° C inside the chamber at the area of the pups. The ISO was vaporized into room air with 30% oxygen. The concentration of gases was measured by a gas analyzer (Datex Cardiocap II, Datex-Ohmeda, Madison, WI) via a sensing device placed in the flow chamber. The carbon dioxide level in the chamber was maintained at less than 1%.

Thirty min following cessation of gas exposure and confirmation of recovery from anesthesia, the pups were returned to their dams. The dams attended to the pups equally. Subsequently, 2.5 hr later (6 hr following initiation of ISO exposure), pups were deeply anesthetized via intraperitoneal (i.p.) injection of 0.03 ml of sodium pentobarbital and perfused with a heparinized saline flush followed by 4% paraformaldehyde in a 0.1M sodium phosphate buffer. Immediately following perfusion, the whole brain was removed

from each pup and post-fixed in perfusate for 4 days. Each brain was rinsed in distilled water, laterally bisected using a razor blade, and one hemisphere blocked in Agar in preparation for sectioning. One hr later, when the Agar cooled to provide a supportive medium, the hemispheres were serially sectioned (70 μm in thickness) on a Vibratome and stored in phosphate buffered saline (PBS).

Activated caspase-3 immunohistochemistry. Immunohistochemical techniques were used to detect apoptotic neurodegeneration through AC3 staining as previously described (Young et al., 2005). Every eighth vibratome section was chosen in an unbiased, systematically random sampling manner according to the principles of stereology. This permits sampling 8 to 10 sections from half of each brain. Any remaining Agar was removed from the sections, which were then placed in 0.01 M PBS. The sections were quenched in methanol containing 3% hydrogen peroxide, then washed 3 times for 5 min each in 0.01 M PBS, followed by a 60 min incubation in blocking solution (2% bovine serum albumin + 0.2% dry milk in 0.1% triton-X PBS), and then incubated overnight in rabbit anti-active caspase-3 antiserum in blocking solution (1:1000 dilution; Cell Signaling Technology, Beverly, MA). The next day the sections were washed 3 times for 5 min in 0.01 M PBS, incubated for 60 min in goat anti-rabbit secondary antiserum (1:200 dilution), and washed, again, 3 times for 5 min each in 0.01 M PBS. The sections were then reacted in the dark with ABC reagents (standard Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) for 60 min, and washed, once again, 3 times for 5 min each in 0.01 M PBS. Finally, the sections were incubated in VIP reagents (Vector VIP substrate kit for peroxidase, Vector Labs, Burlingame, CA) for approximately 5 min. The sections were mounted onto 75 x 25

Superfrost Plus slides, dehydrated with a series of graded ethanols and Citrisolv, and cover-slipped with Permount and 60 x 24 cover slips.

Density quantification. Quantification of apoptotic neurodegeneration was conducted as previously described (Young & Olney, 2006). A stereology system was used consisting of the following components: Stereo Investigator (MicroBrightField, Inc., Colchester VT) on a Pentium III PC, connected to a Prior Optiscan motorized stage (ES103 XYZ system, Prior Scientific Inc., Rockland MA) mounted on a Nikon Labophot-2 microscope. The sections were imaged and, using the computer software, the boundaries of the brain regions of interest (caudate, cerebral cortex, dorsal hippocampus, and thalamus) were traced manually according to a mouse brain atlas on three sections from each brain and the area then calculated. Counts were performed across the entire extent of the identified regions. The population estimator function of Stereo Investigator was used for the manual marking of each profile positively stained for AC3 during counting to ensure that no profile was missed or counted twice. To obtain an estimate of profile density in the current experiment, the total number of AC3-positive profiles counted in each brain was divided by the total area within which these profiles were present for each pup. To obtain an estimate of profile density for the total brain area examined, the total numbers of AC3-positive profiles counted in the all areas was divided by the total area within which these profiles were present for each pup.

Statistical Analyses

All statistical analyses were performed using the SPSS statistical program for PC computers, version 18. Means and standard errors were computed for each measure. Factorial ANOVAs, included repeated measures ANOVAs, were used where appropriate.

The appropriate correction, Huynh-Feldt or Greenhouse-Geisser, was applied to violations of the sphericity assumption within a rmANOVA. With a statistically significant interaction between main factors, simple main effects were calculated to provide clarification of statistically significant between-exposure and within-exposure differences. Multiple comparisons were Bonferroni adjusted. Tukey's HSD method was used as a post hoc test. Probability value for all analyses was $p < .05$.

Results

Total Brain

To investigate the age at which a single ISO exposure produces the most acute damage, a 2 x 3 ANOVA was performed on density of AC3-positive profiles within the total brain area with exposure and age (PND 3 vs. PND 5 vs. PND 7) as the main factors. A statistically significant exposure x age interaction, $F(2, 36)=3.793$, $p=.032$, was further analyzed for simple main effects (Figure 2). ISO exposure increased AC3-positive profile densities compared to room air on each age PND 3, PND 5, and PND 7, $F(1, 36)=6.13 – 36.448$, $p<.019$. The only within-exposure differences yielded by the analyses were those within the ISO group between ages, $F(2, 36)=17.433$, $p=.000$, such that ISO induced a greater AC3-positive profile density on both PND 3 and PND 5 compared to PND 7, $p=.001$ and $p=.000$, respectively.

A statistically significant main effect of exposure, $F(1, 36)=56.378$, $p=.000$, revealed ISO induced a greater density of AC3-positive profiles across all ages compared to room air.

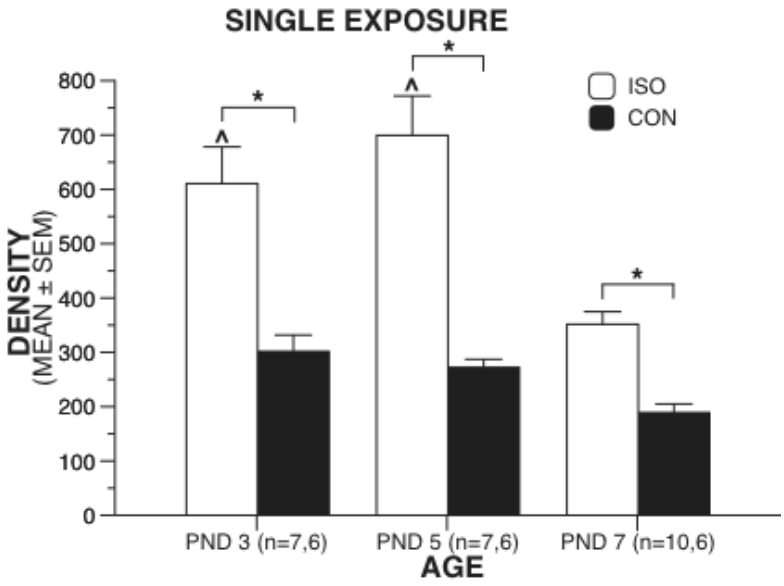

Figure 2. Exposure to 1.5% ISO for 3 hr induced greater densities of AC3-positive profiles within the total brain area compared to controls on all ages PND 3, 5, and 7. In general, ISO exposure increased density of AC3-positive profiles compared to controls. Between exposure groups, greater densities were observed following ISO exposure compared to controls on all ages, PND 3, 5, and 7. Within the ISO exposure group, greater densities were observed on PND 3 and 5 compared to PND 7. * Denotes a statistically significant difference between exposure groups, $p<.019$. **^** Denotes a statistically significant difference from ISO exposure on PND 7, $p<.002$.

A 2 x 3 ANOVA with age at staining (PND 5 vs. PND 7) and number of ISO exposures (zero vs. single vs. double) as main factors was performed on density of AC3-positive profiles within the total brain area to clarify the neuroapoptotic influence of different numbers of ISO exposures at different ages. A statistically significant age x number of exposures interaction was revealed, $F(2, 50)=10.181$, $p=.000$ (Figure 3). Further analysis for simple main effects yielded differences between number of exposures on PND 5 and PND 7, $F(2, 50)=15.349 – 37.780$, $p=.000$. On PND 5, single and double exposures induced greater densities compared to zero exposures, both $p=.000$. A single exposure on PND 5 also induced a greater density of AC3-positive profiles compared to double exposures, $p=.006$. On PND 7, single and double exposures induced greater densities of AC3-positive profiles compared to zero exposures, $p<.005$. A single exposure on PND 5 induced a greater density of AC3-positive profiles compared to a

single exposure on PND 7, $F(1, 50)=39.738$, $p=.000$. No differences were revealed between a double exposure on PND 5 and PND 7 or between zero exposures on PND 5 and PND 7.

Post hoc comparisons of a statistically reliable main effect of number of exposures, $F(2, 50)=45.631$, $p=.000$, revealed greater AC3-positive profile densities overall following a single and a double exposure ISO compared to zero exposures, $p=.000$.

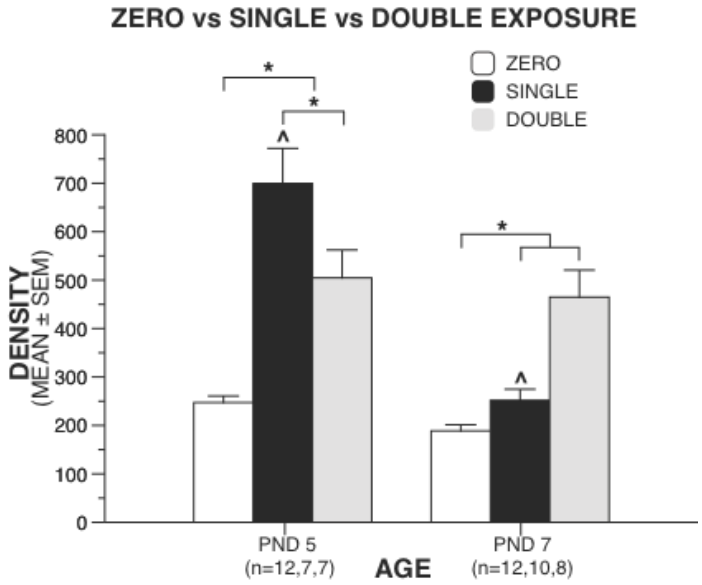

Figure 3. Single and double exposures to 1.5% ISO for 3 hr on PND 5 and 7 induced greater densities of AC3-positive profiles within the total brain area compared to controls. Single and double ISO exposures increased density of AC3-positive profiles compared to zero exposures regardless of age at exposure. On PND 5, a single exposure induced greater densities compared to a double or zero exposures. On PND 7, single and double exposures induced greater densities compared to zero exposures, with no differences between single and double exposures. A single exposure on PND 5 induced greater densities compared to a single exposure on PND 7.* Denotes a statistically significant difference between the numbers of exposures on that age, $p<.007$. **^** Denotes a statistically significant difference between a single exposure on PND 5 or PND 7, $p<.001$.

To investigate the differences in AC3-positive profile densities within the total brain area following 0, 1, 2 or 3 ISO exposures on PND 7, a one-way ANOVA was conducted with number of ISO exposures (0 vs. 1 vs. 2 vs. 3) as the main factor. A statistically significant main effect, $F(3, 40)=26.605$, $p=.000$, was further analyzed using

post hoc comparisons (Figure 4). AC3-positive profile densities were greater following 2 exposures compared to 3, 1, or 0 exposures, $p<.034$. Densities were also greater following 1 exposure compared to 0 exposures, $p=.000$.

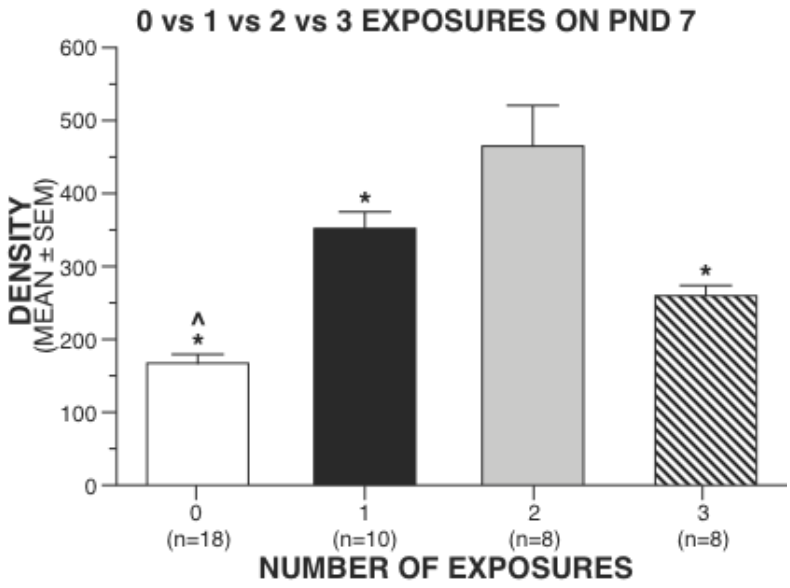

Figure 4. The density of AC3-positive profiles observed in the total brain on PND 7 was dependent on the number of exposures to 1.5% ISO for 3 hr. A greater density was observed following two exposures compared to 3, 1, or 0 exposures. A greater density was observed following 1 exposure compared to zero exposures. * Denotes a statistically significant difference from 2 ISO exposures on PND 7, $p<.034$. **^** Denotes a statistically significant difference from 1 ISO exposure on PND 7, $p=.000$.

Caudate, Cerebral Cortex, Dorsal Hippocampus, and Thalamus

To quantitatively compare the age at which ISO exposure produces the most acute damage within each area of the brain examined, a 2 x 3 x 4 rmANOVA was performed on density of AC3-positive profiles with exposure and age (PND 3 vs. PND 5 vs. PND 7) as the main factors and area (caudate vs. cortex vs. hippocampus vs. thalamus) as the within-subjects repeated measure. In general, ISO exposure induced a greater density of AC3-positive profiles compared to room air exposure, $F(1, 36)=47.148$, $p=.000$, as indicated by a statistically significant main effect of exposure yielded by the main rmANOVA (Figure 5).

The analysis yielded a statistically significant three-way interaction between exposure, age, and area, $F(6, 108)=5.734$, $p=.000$, which was further analyzed for simple main effects. Between-subjects analyses revealed differences in densities between exposure groups on different ages within each area. Exposure to ISO on PND 3 induced a greater density of AC3-positive profiles compared to room air exposure in the cortex, hippocampus, and thalamus, $F(1, 36)=5.645 – 38.141$, $p<.023$. Exposure to ISO on PND 5 induced a greater density of AC3-positive profiles compared to room air in the caudate, cortex, hippocampus, and thalamus, $F(1, 36)= 8.653 – 28.517$, $p<.007$. Exposure to ISO on PND 7 induced a greater density of AC3-positive profiles compared to room air exposure within only the cortex, $F(1, 36)=14.705$, $p=.000$.

Further between-subjects analyses revealed differences in densities between ages following ISO exposure within different areas. Within the caudate, $F(2, 36)=24.925$, $p=.000$, ISO exposure on PND 3 induced a greater density of AC3-positive profiles compared to ISO exposure on PND 7, $p=.013$. In addition, ISO exposure on PND 5 induced a greater density of AC3-positive profiles in the caudate compared to that on PND 3 and PND 7, $p<.003$. Within the cortex, $F(2, 36)=5.348$, $p=.009$, ISO exposure on PND 5 induced a greater density of AC3-positive profiles compared to that on PND 3, $p=.008$. Within the hippocampus, $F(2, 36)=16.192$, $p=.000$, ISO exposure on PND 3 and PND 5 induced greater AC3-positive profile densities compared to ISO exposure on PND 7, $p<.003$. Finally, within the thalamus, $F(2, 36)=27.656$, $p=.000$, ISO exposure on PND 3 induced a greater density of AC3-positive profiles compared to ISO exposure on PND 5 and PND 7, $p<.003$. In addition, ISO exposure on PND 5 induced a greater density of AC3-positive profiles in the thalamus compared to that on PND 7, $p=.007$.

Within-subjects analyses for simple main effects of the three-way interaction yielded differences in densities between areas on different ages within each exposure group. On PND 3, ISO exposure induced differences in AC3-positive profile densities, $F(3, 34)=17.992$, $p=.000$, such that caudate & hippocampus & thalamus > cortex, all $p=.000$. Also on PND 3, difference between densities of AC3-positive profiles were observed within the control mice, $F(3, 34)=9.512$, $p=.000$, such that caudate & hippocampus > cortex, as well as hippocampus > thalamus, $p<.020$. On PND 5, ISO exposure induced differences in AC3-positive profile densities, $F(3, 34)=26.720$, $p=.000$, such that caudate > cortex & hippocampus & thalamus, all $p=.000$. Also on PND 5, difference between densities of AC3-positive profiles were observed within the control mice, $F(3, 34)=4.594$, $p=.008$, such that caudate > cortex & thalamus, $p<.028$. On PND 7, ISO exposure induced differences in AC3-positive profile densities, $F(3, 34)=3.133$, $p=.038$, such that the cortex > thalamus, $p=.033$.

The main rmANOVA also yielded a statistically significant exposure x age interaction, $F(2, 36)=5.565$, $p=.008$. Further analyses for simple main effects revealed ISO exposure induced greater AC3-positive profile densities compared to room air exposure on PND 3 and PND 5, $F(1, 36)=19.958 – 33.298$, $p=.000$. Moreover, exposure to ISO on PND 3 and PND 5 yielded greater densities of AC3-positive profiles compared to ISO exposure on PND 7, $F(2, 36)=25.131$, $p=.000$.

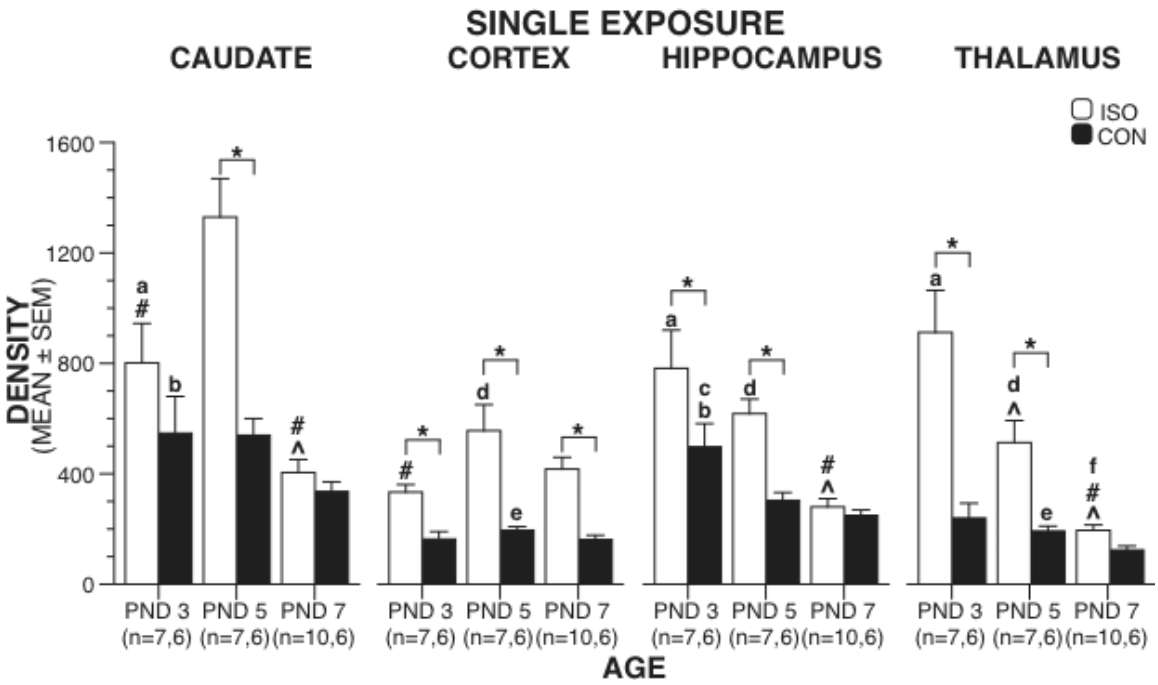

Figure 5. Exposure to 1.5% ISO for 3 hr induced differences in densities of AC3-positive profiles within the caudate, cortex, hippocampus, and thalamus compared to controls on all ages PND 3, 5, and 7. In general, ISO exposure increased density of AC3-positive profiles compared to controls. ISO exposure increased densities compared to controls on PND 3 in the cortex, hippocampus, and thalamus, on PND 5 in all 4 areas, and on PND 7 in the cortex. Among ISO mice, densities were greater in the caudate such that PND 3 > 7, and PND 5 > 3 & 7; in the cortex on PND 5 > 3; in the hippocampus on PND 3 & 5 > 7; and in the thalamus on PND 3 > 5 > 7. Greater densities were observed such that caudate & hippocampus & thalamus > cortex in PND 3 ISO mice; caudate & hippocampus > cortex, and hippocampus > thalamus in PND 3 controls; caudate > cortex & hippocampus & thalamus in PND 5 ISO mice; caudate > cortex & thalamus in PND 5 controls; and cortex > thalamus in PND 7 ISO mice. Between exposure groups, greater densities were observed following ISO exposure compared to controls on ages PND 3 and PND 5. Within the ISO mice, densities were greater on PND 3 and 5 relative to PND 7.

* Denotes a statistically significant difference between exposure groups, p=.000. **^** Denotes a statistically significant difference from ISO exposure on PND 3 within respected area, p<.014. **#** Denotes a statistically significant difference from ISO exposure on PND 5, p<.009. **a** Denotes a statistically significant difference from ISO exposure on PND 3 in the cortex, p=.000. **b** Denotes a statistically significant difference from controls on PND 3 in the cortex p<.003. **c** Denotes a statistically significant difference from controls on PND 3 in the thalamus, p=.020. **d** Denotes a statistically significant difference from ISO exposure on PND 5 in the caudate, p=.000. **e** Denotes a statistically significant difference from controls on PND 5 in the caudate, p<.028. **f** Denotes a statistically significant difference from ISO exposure on PND 7 in the cortex, p=.033.

A 2 x 3 x 4 rmANOVA with age at staining (PND 5 vs. PND 7) and number of ISO exposures (zero vs. single vs. double) as main factors and area (caudate vs. cortex vs. hippocampus vs. thalamus) was performed on density of AC3-positive profiles to clarify the neuroapoptotic influence of different numbers of ISO exposures on different ages. A

statistically significant three-way age x exposure x area interaction, $F(4.808, 120.197)=6.104$, $p=.000$, was further analyzed for simple main effects (Figure 6). Differences were observed between exposure levels on PND 5 within the caudate, $F(2, 50)=24.239$, $p=.000$, such that single > double > zero, $p<.008$; within the cortex, $F(2, 50)=17.472$, $p=.000$, such that single > double > zero, $p<.048$; within the hippocampus, $F(2, 50)=17.468$, $p=.000$, such that single > zero & double $p<.005$; and within the thalamus, $F(2, 50)=15.571$, $p=.000$, such that single & double > zero, $p=.000$. Differences were observed between exposure levels on PND 7 within the caudate, $F(2, 50)=3.346$, $p=.043$, such that double > zero, $p=.045$; cortex, $F(2, 50)=19.412$, $p=.000$, such that single & double > zero, $p=.000$; and within the hippocampus, $F(2, 50)=4.927$, $p=.011$, such that double > zero, $p=.009$.

Further analysis for the between-subjects of the simple main effects of the three-way interaction revealed greater densities on PND 5 compared to PND 7 within the all brain areas following a single ISO exposure, $F(1, 50)=4.177 – 56.534$, $p<.047$. Densities also were greater on PND 5 compared to PND 7 within the thalamus following double exposures, $F(1, 50)=11.058$, $p=.002$. In contrast, densities were greater on PND 7 compared to PND 5 within the cortex following double exposures, $F(1, 50)=4.874$, $p=.032$.

Analysis for the within-subjects simple main effects of the three-way interaction revealed differences in AC3-positive profile densities between brain areas on PND 5 and PND 7 across all three levels of exposure. A single ISO exposure on PND 5, $F(3, 48)=43.435$, $p=.000$, induced a greater density within the caudate compared to all other areas, $p=.000$. A single ISO exposure on PND 7, $F(3, 48)=5.955$, $p=.002$, induced a

greater density within the caudate and cortex compared to the thalamus, within the cortex compared to the hippocampus, $p<.033$. Double ISO exposures on PND 5, $F(3, 48)=11.404$, $p=.000$, induced a greater density within the caudate compared to all other areas, $p=.000$. Double ISO exposures on PND 7, $F(3, 48)=13.784$, $p=.000$, induced a greater density within the caudate, cortex, and hippocampus compared to the thalamus, as well as within the caudate compared to the hippocampus, $p<.032$.

The main analysis yielded a statistically significant two-way interaction between exposures and age, $F(2, 50)=12.917$, $p=.000$. Further analysis for simple main effects yielded significant differences in densities following a single ISO exposure on PND 5 compared to that on PND 7, $F(1, 50)=54.209$, $p=.000$. On PND 5, $F(2, 50)=36.279$, $p=.000$, single and double ISO exposures increased AC3-positive profile densities compared to zero exposure, $p=.000$. A single exposure on PND 5 also increased densities compared to double ISO exposures, $p=.004$. On PND 7, double ISO exposures increased densities compared to zero, $F(2, 50)=9.456$, $p=.000$.

A statistically reliable two-way exposure x area interaction was revealed by the main rmANOVA, $F(4.808, 120.197)=3.469$, $p=.006$ (Figure 7). Further between-subjects analysis revealed both single and double ISO exposures induced greater AC3-positive profile densities compared to zero exposures within the caudate, cortex, hippocampus, and thalamus, $F(2, 50)=13.100 – 33.155$, $p=.000$. Within-subjects analysis revealed greater densities within the caudate compared to all other areas at all exposure levels, zero, single, and double, $F(3, 48)=20.128 – 38.032$, $p=.000$. Greater densities also were observed within the hippocampus compared to the thalamus following zero exposures, $F(3, 48)=24.906$, $p=.000$.

The main rmANOVA yielded a statistically significant main effect of exposure, $F(2, 50)=35.696$, $p=.000$, Post hoc analyses of the main effect of exposure revealed greater densities following single and double exposures compared to zero exposures, both $p=.000$.

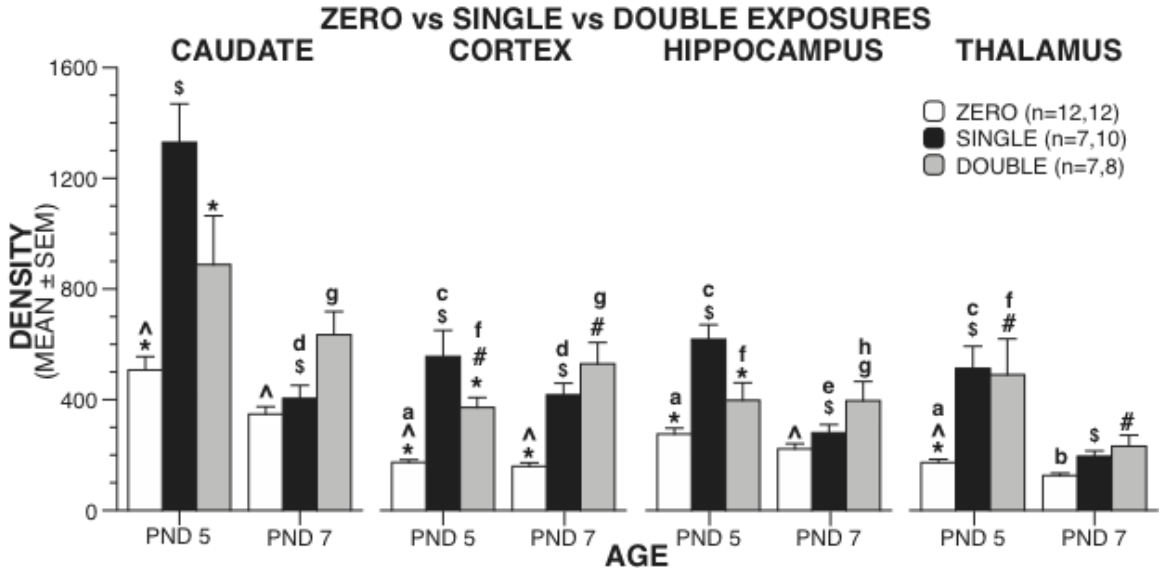

Figure 6. A single and a double exposure to 1.5% ISO for 3 hr induced differences densities of AC3-positive profiles within all brain areas compared to controls on PND 5 and PND 7. In general, both a single and a double exposure to ISO induce greater densities compared to zero exposures. With all brain areas combined, a single ISO exposure on PND 5 induces a greater density compared to that on PND 7. A single ISO exposure on PND 5 produces densities greater compared zero or double ISO exposures. Double ISO exposures on both PND 5 and 7 induce greater densities compared to zero exposures. On PND 5, differences in densities occur between the levels of ISO exposure within every brain area. On PND 7, density differences occur between levels of ISO exposure within the caudate, cortex, and hippocampus. Differences in densities occur within the caudate, hippocampus, and thalamus between PND 5 and 7 following a single ISO exposure. Densities are greater following a single exposure on PND 5 compared to PND 7 within all brain areas. Densities are greater following a double exposure on PND 5 compared to PND 7 within the thalamus. In contrast, densities are greater following a double exposure on PND 7 compared to PND 5 within the cortex. At each level of ISO exposure, density differences occur between brain areas on both PND 5 and PND 7.

* Denotes a statistically significant difference from a single ISO exposure within the respective area, $p=.000$. **^** Denotes a statistically significant difference from double ISO exposures within the respective area, $p<.003$. **$** Denotes a statistically significant difference between ages after a single ISO exposure within respective area, $p<.047$. **#** Denotes a statistically significant difference between ages after double ISO exposures within respective area, $p<.033$. **a** Denotes a statistically significant difference from zero ISO exposures on PND 5 in the caudate, $p<.007$. **b** Denotes a statistically significant difference from zero ISO exposures on PND 7 in the caudate, $p=.002$. **c** Denotes a statistically significant difference from a single ISO exposure on PND 5 in the caudate, $p=.000$. **d** Denotes a statistically significant difference from a single ISO exposure on PND 7 in the thalamus, $p<.015$. **e** Denotes a statistically significant difference from a single ISO exposure on PND 7 in the cortex, $p=.032$. **f** Denotes a statistically significant difference from double ISO exposures on PND 5 in the caudate, $p=.000$. **g** Denotes a statistically significant

difference from double ISO exposures on PND 7 in the thalamus, $p<.012$. **h** Denotes a statistically significant difference from double ISO exposures on PND 7 in the caudate, $p=.031$.

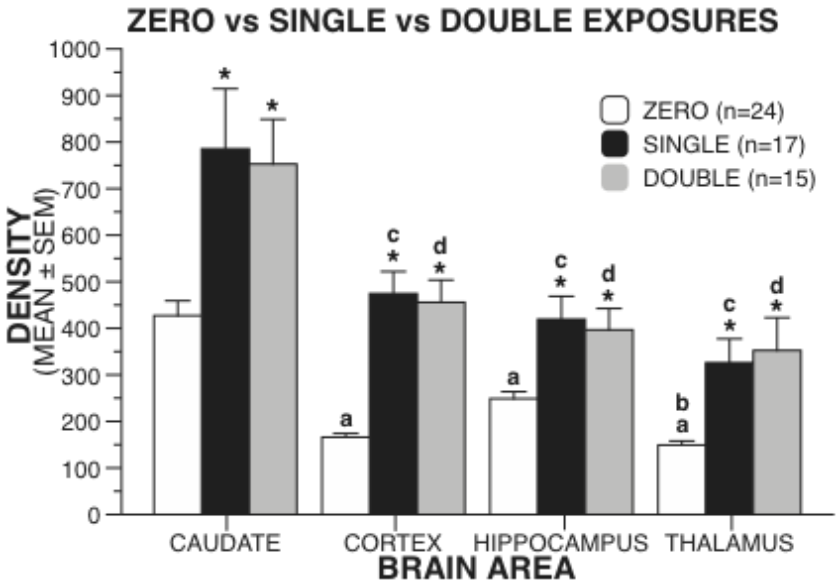

Figure 7. Single and double exposures to 1.5% ISO for 3 hr increase AC3-positive profile densities compared with zero exposures in all brain areas. Within all brain areas, single and double ISO exposures increased densities compared to zero exposures. Differences were observed between brains areas following each level of ISO exposure, zero, single and double. * Denotes a statistically significant difference from single and double ISO exposures, $p<.002$. **a** Denotes a statistically significant difference from zero ISO exposures in the caudate, $p<.003$. **b** Denotes a statistically significant difference from zero ISO exposures in the hippocampus $p=.007$. **c** Denotes a statistically significant difference from a single ISO exposure in the caudate, $p=.000$. **d** Denotes a statistically significant difference from double ISO exposure in the caudate, $p=.000$.

To investigate the differences in AC3-positive profile densities following 0, 1, 2 or 3 ISO exposures, a 4 x 4 rmANOVA was conducted with number of ISO exposures (0 vs. 1 vs. 2 vs. 3) as the main factor and area (caudate vs. cortex vs. hippocampus vs. thalamus) as the within-subjects repeated measure. A statistically significant exposure x area interaction, $F(5.254, 70.054)=4.280$, $p=.002$, was revealed (Figure 8).

Between-subjects analysis of simple main effects revealed differences between numbers of exposures within each brain area. Within the caudate, $F(3, 40)=7.724$, $p=.000$, 2 exposures induced greater densities compared to 0, 1, and 3 exposures, $p<.024$. Within the cortex, $F(3, 40)=24.391$, $p=.000$, greater densities were observed with 1, 2, and 3 exposures compared to 0 and with 2 exposures compared to 3, $p<.033$. Within the

hippocampus, $F(3, 40)=6.224$, $p=.001$, 2 exposures increased densities compared to 0 and 3 exposures, $p<.023$. Within the thalamus, $F(3, 40)=9.076$, $p=.000$, greater densities were observed with 1 exposure compared to 0 and with 2 exposures compared to 0 and 3, $p<.010$.

Within-subjects analysis revealed differences between brain areas with each number of ISO exposures. Following 1 ISO exposure, $F(3, 38)=31.809$, $p=.000$, greater differences were observed in the caudate compared to the thalamus, in the cortex compared to the hippocampus and thalamus, and in the hippocampus compared to the thalamus, $p<.046$. Densities were greater following 2 ISO exposures, $F(3, 38)=66.803$, $p=.000$, in the caudate and cortex compared to the hippocampus and thalamus, and in the hippocampus compared to the thalamus, $p<.004$. Following 3 ISO exposures, $F(3, 38)=25.126$, $p=.000$, densities were greater in the caudate, cortex, and hippocampus compared to the thalamus, $p<.017$.

The main analysis also yielded a statistically significant main effect of exposure, $F(3, 40)=19.186$, $p=.000$, indicating, overall, densities were greater following 2 ISO exposures compared to 0, 1, or 3, and following 1 exposure compared to 0 exposures, $p<.017$.

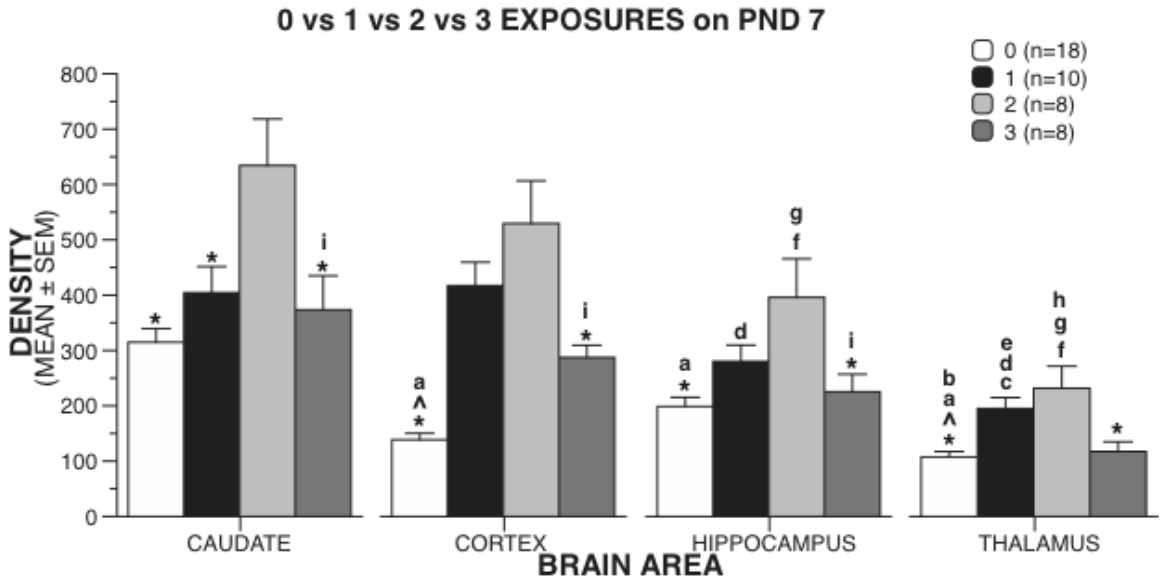

Figure 8. The density of AC3-positive profiles observed on PND 7 in each area of the brain was dependent on the number of exposures to 1.5% ISO for 3 hr. One or 2 exposures increased AC3-positive profile densities compared to 0 exposures on PND 7. Two exposures increased densities compared to 1 or 3 exposures. Within each brain area differences in densities were observed between the levels of ISO exposure. Differences in densities were observed between brain areas within each level of ISO exposure, as well. * Denotes a statistically significant difference from 2 ISO exposures. **^** Denotes a statistically significant difference from 1 ISO exposure.

a Denotes a statistically significant difference from 0 ISO exposures in the caudate, $p<.030$. **b** Denotes a statistically significant difference from 0 ISO exposures in the hippocampus, $p=.001$. **c** Denotes a statistically significant difference from 1 ISO exposure in the caudate, $p=.000$. **d** Denotes a statistically significant difference from 1 ISO exposure in the cortex, $p<.002$. **e** Denotes a statistically significant differences from 1 ISO exposure in the hippocampus, $p=.045$. **f** Denotes a statistically significant difference from 2 ISO exposures in the caudate, $p<.002$. **g** Denotes a statistically significant difference from 2 ISO exposures in the cortex, $p<.004$. **h** Denotes statistically significant difference from 2 exposures in the hippocampus, $p<.000$. **i** Denotes a statistically significant difference from 3 ISO exposures in the thalamus.

Discussion

This experiment was designed to investigate ISO influence on neuroapoptosis in the postnatal mouse brain at different ages and following different numbers of exposures during the brain growth spurt period of development. In agreement with the first hypothesis, a single ISO exposure increased acute neuroapoptosis in whole brains compared to controls on each age at exposure. A single exposure to ISO on PND 3 or PND 5 induced a greater neuroapoptotic response than that on PND 7. No differences in neuroapoptosis were observed in whole brains between ages of the control groups.

Analyses of the influence of treatments in the separate brain areas indicated an increase in neuroapoptosis following ISO exposure at each age PND 3, PND 5, and PND 7 only in the cerebral cortex. Within the hippocampus and the thalamus, ISO exposure on PND 3 and PND 5 increased neuroapoptosis. Within the caudate, ISO exposure increased neuroapoptosis only on PND 5.

Differences were also found in neuroapoptotic response following ISO exposure at different ages in each area. As hypothesized, ISO exposure induced the greatest neuroapoptotic response in the thalamus on PND 3 and in the caudate on PND 5. In contrast to the hypothesis, ISO exposure on PND 5 induced a large increase in neuroapoptosis in the cerebral cortex, however, this was not different from that on PND 7, only from that on PND 3. ISO induced a greater neuroapoptotic response in the hippocampus on PND 3 compared to PND 7, but not that observed on PND 5.

In support of the third hypothesis, multiple ISO exposures altered the pattern of neuroapoptosis (Figure 9). An increase in neuroapoptotic response, however, was only observed following multiple exposures on PND 7 within the dorsal hippocampus. The impact of multiple versus single exposures on the neuroapoptotic response is discussed further in the later General Discussion section.

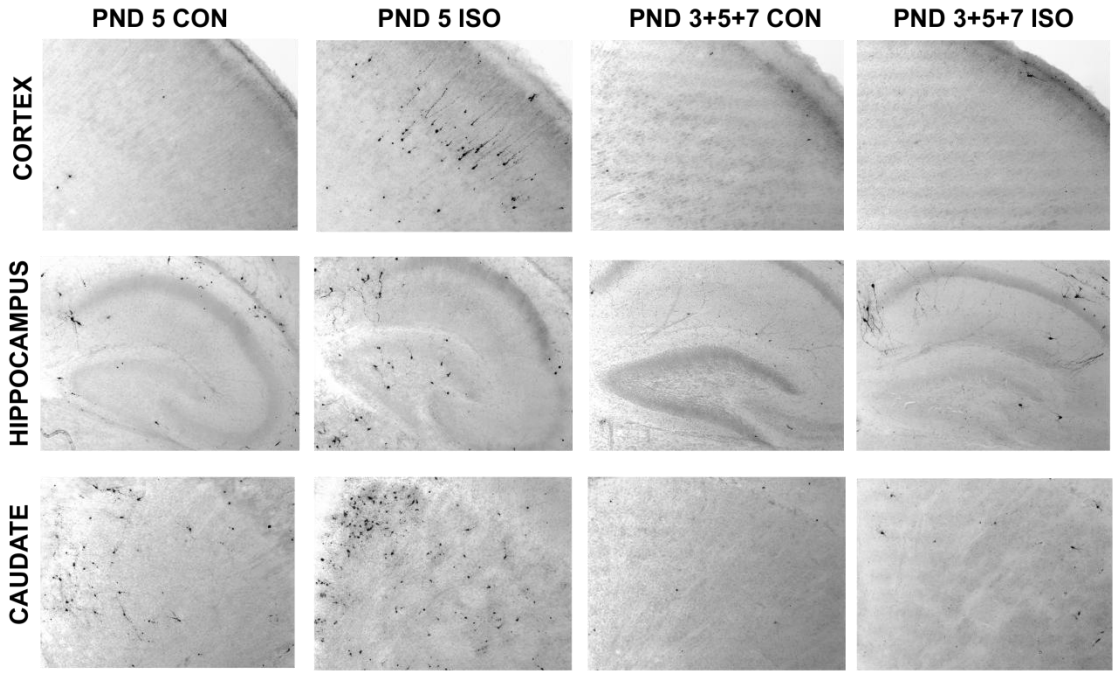

Figure 9. The density of profiles stained by AC3-immunohistochemistry (small dark spots) in brains exposed to ISO neonatally was larger than the densities in controls brains. Multiple ISO exposures altered the observed amount of AC3-positive profiles relative to a single ISO exposure.

EXPERIMENT 2

An estimated 6 million children, including 1.5 million infants, undergo anesthesia exposure for surgeries each year (DeFrances, Cullen, & Kozak, 2007). Many require multiple surgeries to correct a congenital problem. Clinical evidence suggests multiple exposures to anesthesia before the age of four are associated with an increased risk of later learning disabilities (Flick et al., 2011; Wilder et al., 2009). The general anesthetic ISO is commonly used in pediatric anesthesia (Lonnquist, 2002), and, for that reason, it is important to investigate possible long-term adverse functional outcomes of developmental exposure to ISO.

Previous research using rodent models of postnatal ISO exposure reveals deficits predominately within the cognitive domain. Resultant disruptions in spatial learning and memory in MWM and RAM paradigms and in contextual fear-conditioning and working memory during object recognition have all been observed in the rat (Rothstein et al., 2008; Stratmann, May, et al., 2009; Zhu et al., 2010). Cognitive impairments following neonatal ISO exposure are less pronounced in the mouse, although decreased fear-conditioning and impaired reversal place learning have been reported (Kodama et al., 2011; Zhu et al., 2010). Research also suggests emotionality disturbances may result from developmental ISO exposure (Rothstein et al., 2008).

Experiment 1 confirmed postnatal ISO exposure enhances neuroapoptosis in the in the mouse brain between the ages of PND 3 – 7, suggesting a possible alteration of neural circuitry. The purpose of the second experiment was to investigate the longitudinal impact of this ISO-induced increase in neuroapoptosis on behavior. Mouse pups were exposed to ISO on PND 3+5+7, and neuromotor, cognitive, emotional and social functioning were assessed beginning in the juvenile stage and continuing into adulthood. The multiple dosing protocol was based on a study of children. Clinical studies reported that multiple anesthesia exposures before the ages of 2 and 4 increased the risk of LD and the risk increased with a longer cumulative duration of anesthesia (Flick et al., 2011; Wilder et al., 2009). The multiple dosing protocol allowed for the investigation of the influence of multiple anesthesia exposures on behavior in an *in vivo* mouse model.

We hypothesized the greatest behavioral disruption would occur in the cognitive domain. This was based on previous research and the extent of neuroapoptotic

abnormality following postnatal ISO exposure in the cortex and hippocampus in Experiment 1.

We included measures of socio-emotional behaviors because few studies in the literature have included measures of emotional behaviors and, especially, social behaviors. In Experiment 1, increased neuroapoptosis was observed following ISO exposure the hippocampus and cortex. These two areas greatly interact with areas involved in emotional and social behaviors (Roxo, Franceschini, Zubaran, Kleber, & Sander, 2011). For this reason, we hypothesized that multiple postnatal ISO exposures would adversely affect both emotional and social behavior in the mouse.

A substantial increase in neuroapoptosis was observed in Experiment 1 in the cortex, caudate, and thalamus. These areas are heavily involved in general neuromotor functioning. It is not clear if postnatal ISO exposure would influence later general neuromotor functioning given that previous research has not demonstrated deficits in these domains. Thus, we included tests of general functioning to serve as controls for the interpretation of the cognitive, emotional, and social behavioral testing.

Methods and Materials

Animals

A total of 35 experimentally naïve C57BL6 mice, 15 females and 20 males, from 5 different litters were used as subjects for this experiment. Age- and litter-matched pups were exposed to room air to serve as controls. All animal housing conditions were the same as in Experiment 1. Upon weaning on PND 21, subject mice were housed (in groups of five) according to gender and random distribution across treatment groups in translucent plastic cages measuring 28.5 cm x 17.5 cm x 12 cm with corncob bedding.

All mice were carefully monitored throughout the experiment for weight and general appearance.

Experimental Design

The second experiment was designed to investigate multiple neonatal ISO exposures on behavior during juvenile, adolescent, and adult stages. The two exposure groups were as follows:

Group 1: PND 3+5+7 ISO exposed (*n* = 19; 9 females, 10 males)
Group 2: PND 3+5+7 litter-matched room air exposed controls (*n* = 16; 6 females, 10 males)

Procedures

Mouse pups were exposed to 1.5% ISO or room air for 3 hr PND 3, 5, and 7. The anesthesia exposure procedure was the same as Experiment 1. Thirty min following cessation of gas exposure and confirmation of recovery from anesthesia the pups were placed back with the dams. Pups were weighed on each day PND 3 – 21, and again on PND 30, 38, 45, and 60. Behavioral testing began immediately following weaning on PND 21, with reactivity to handing testing (Table 1). A 1 hr locomotor activity/exploration test and evaluation on a battery of sensorimotor skills was performed to assess any general activity and exploration or sensorimotor disruptions that may interfere with interpretation of later behavior tests. Morris water maze testing began with cued trials, which served to train the mice to function in the water maze, in terms of their swimming abilities and to determine any nonassociative dysfunctions that would compromise their performance. The mice received acquisition training during the place trials (spatial learning), followed by a 1 min probe trial to evaluate spatial memory retention. Fine motor coordination was evaluated on the rotarod and anxiety-related

behaviors were tested in the EPM. Social approach behaviors of the mice were also assessed. All mice were re-tested in the 1 hr locomotor activity/exploration test, the EPM, and in the social approach paradigm to explore any long-term general activity-related functional abnormalities, emotionality disruptions, or abnormalities in social behaviors resulting from postnatal ISO exposure. Lastly, the mice were assessed in the fear-conditioning paradigm.

Table 1. Tests to evaluate behavioral effects of multiple neonatal ISO exposures.

Behavioral Tests	**Ages at Testing (postnatal days; PND)**
Reactivity to Handling	PND 22-24
1 hr Locomotor Activity	PND 31/PND 137
Sensorimotor Battery	PND 32-33
Morris Water Maze	PND 34-42
Rotarod	PND 45, 49, 53
Elevated Plus Maze	PND 54-56/PND 144-146
Social Approach	PND 60/PND 178
Conditioned Fear	PND 194-196

Behavioral tests.

Reactivity to handling. In order to assess early post-weaning reactivity to being handled the mice were evaluated on 3 separate, consecutive days starting on PND 22. This test also served to habituate the mice to handling. On each assessment day, each mouse was picked up by the tail from its home cage, placed on the wire grid, and its tail was marked with a non-toxic Sharpie pen for identification purposes. During the tail marking, the behavior of the mouse was judged and ranked by three separate observers. The rankings range from 1 to 5, with 1 indicating almost no reactive response to handling and 5 indicating substantial reactivity to handling (Table 2). Each animal received one reactivity score per assessment day from each observer, which was averaged across observers for that assessment day. A single reactivity to handling score per assessment

day, therefore, was analyzed for each mouse.

Table 2. Rankings for reactivity to handling scores.

Score	Behavioral Response to Handling by Mouse
1	Very minimal resistance to being picked up by the tail from the home cage and relatively inactivity while on the grid during tail marking.
2	Minimal resistance to being picked up by the tail from the home cage but briefly active while on the grid during tail marking.
3	Some resistance to being picked up by the tail from the home cage, but captured quickly or active on the grid the entire duration of tail marking.
4	Resistance to being picked up by the tail from the home cage and extended effort required for capture or jumped around on the grid during tail marking while actively trying to escape the handler.
5	Resistance to being picked up by the tail from the home cage and extended effort required to be captured and jumped around on the grid during tail marking or successfully escaped the handler or bit the handler.

Locomotor activity. A 1 hr locomotor activity/exploration test was conducted to assess the general activity, exploratory behavior, and emotionality of the mice on PND 31. This test also served as a control test to identify any differences in general activity that may interfere with the interpretation of cognitive, social, and/or emotionality tests. The mice were evaluated over a 1 hr period in transparent enclosures (47.6 x 25.4 x 20.6 cm high rat cages) that were surrounded by metal frames containing 4 x 8 matrices of photobeam pairs as previously described (Wozniak et al., 2004). A computer software program (MotorMonitor, Hamilton-Kinder, LLC, Poway, CA) quantified horizontal and vertical beam breaks as ambulations and rearings, respectively, in a 33 x 11 cm central zone and a bordering 5.5 cm peripheral zone. General activity variables (total ambulations, rearings, time at rest) along with measures of emotionality, including time spent, distance traveled and entries made into the central zone, as well as distance traveled in the peripheral zone were analyzed. Each enclosure was cleaned with 75% ethanol solution between each mouse.

All mice were re-tested in the 1 hr locomotor activity/exploration test on PND 137 to explore any changes in general activity from the juvenile stage to adulthood. All testing conditions remained the same as those used initially.

***Sensorimotor battery*.** Two days following completion of the 1 hr locomotor activity testing, PND 32 – 33, balance, strength, and coordination were evaluated by a battery of sensorimotor measures using previously published procedures (Wozniak et al., 2004). The battery included walking initiation, ledge, platform, pole, and inclined and inverted screen tests. An observer manually recorded time in sec and hundredths of a sec using a stopwatch for each test. Two trials were conducted for each test and the average of the two yielded a single time in sec and hundredths of a sec, which was used in the analyses. To avoid exhaustion effects, the order of the tests during the first set of trials was reversed for the second set of trials. The order of the tests was not counterbalanced between animals so that every animal experienced each test under the same conditions. All tests lasted a maximum of 60 s, except for the pole test, which lasted a maximum of 120 s. The tests are described below.

Walking initiation. This test assessed the time taken by a mouse to move out of a small area. The mouse was placed on a flat surface inside a square measuring 21 cm x 21 cm, marked on the surface of a black tabletop with white tape. The time for the mouse to leave the square was recorded, i.e. all four limbs crossed out of the square at the same time.

Balance. Basic balance ability was assessed by the performance on the ledge and platform tests. The ledge test required the mouse to balance on a Plexiglas ledge, measuring 0.75 cm wide and standing 30 cm high, and the time the mouse remained on

the ledge was recorded. During the platform test, the mouse used basic balance ability to remain on a wooden platform measuring 1.0 cm thick and 3.0 cm in diameter and elevated 47 cm above the floor. The time the mouse was able to balance on the platform was recorded.

Fine motor coordination. The pole test was used to evaluate fine motor coordination. The mouse was placed head upward on a vertical pole with a finely textured surface and the time taken by the mouse to turn downward 180° and climb to the bottom of the pole up was recorded.

Strength and coordination. The 60°, 90°, and inverted screen tests assessed a combination of coordination and strength. The mouse was placed head oriented downward in the middle of a mesh wire grid measuring16 squares per 10 cm, elevated 47 cm and inclined to 60° or 90°. The time required by the mouse to turn upward 180° and climb to the top of the screen was recorded. For the inverted screen test, the mouse was placed head oriented downward in the middle of a mesh wire grid measuring16 squares per 10 cm, elevated 47 cm, and, when it was determined the mouse has a proper grip on the screen, it was inverted to 180°. The time the mouse was able to hold on to the screen without falling off was recorded.

***Morris water maze*.** Beginning the day following completion of the sensorimotor battery, PND 34, the mice were tested in the Morris water maze to assess spatial learning and memory. The protocol included three types of trials: cued (visible platform, varied location), place (submerged platform, constant location), and probe (platform removed) as previously described (Wozniak et al., 2004). All trials were conducted in a galvanized steel pool, measuring 120 cm in diameter, and filled with water made opaque with non-

toxic tempura paint. The escape platform was made of PVC and measured 11.5 cm in diameter. A digital video camera connected to a PC computer and the computer software program ANY-maze (Stoelting Co., Wood Dale, IL.) tracked the swimming pathway of the mouse to the escape platform and quantified path length, latency to find escape platform, and swimming speeds of the mice, which were used as outcome variables for cued and place trials. The representation of the pool within the ANY-maze program was divided into quadrants where the target quadrant contained the escape platform.

Cued trials. Each animal received four cued trials on each of 2 consecutive days during which time a wooden dowel rod on top of which was placed a red tennis ball was attached to the escape platform and served as a visual cue. For each cued trial, the escape platform was moved to a different quadrant location within the pool to prevent spatial learning. The mouse was released from the quadrant opposite to the platform location, allowed 60 sec to locate the platform, and, once the platform was found, allowed to sit on it for 30 sec before being returned to its home cage. If the mouse did not locate the platform, a score of 60 sec was recorded, and the animal was placed on the platform for 30 sec before being returned to its home cage. Each trial was separated by approximately 1 hr. Trials were combined into four blocks of two trials for analyses.

Place trials. Place trials began 3 days following completion of the cued trials. Each mouse received two blocks of two consecutive trials on five consecutive days, with an intertrial interval between 30 and 90 sec and approximately 2 hr separating blocks of trials. The escape platform remained in the same quadrant location for all place trials and distal cues were placed on the walls of the room to support spatial learning. The mouse was released from a different location for each of the four trials each day; one release

location near the platform and one far from the platform for each pair of two consecutive trials. Prior to the first place trial, the mouse was placed on the escape platform for 30 sec. The mouse was allowed 60 sec to find the escape platform once released into the pool, and, once the platform was found, allowed to sit on it for 30 sec before being returned to its home cage. If the mouse did not locate the platform, a score of 60 sec was recorded, and the animal was placed on the platform for 30 sec before being returned to its home cage. Trials were combined into five blocks of four trials for analyses.

Probe trial. After the final trial on the fifth day of place trials, one 60 sec probe trial was conducted to assess memory retention of the escape platform location. The escape platform was removed from the pool yet the distal cues remained. The mouse was released into the pool in the quadrant opposite to the former location of the escape platform. At the end of the 60 sec trial, the mouse was removed from the pool and placed back in its home cage. The outcome variables analyzed included swimming speeds, escape platform location crossings, and time spent in each quadrant.

***Rotarod*.** The rotarod (Economex, Columbus Instruments, Columbus, OH) test for coordination and impairment of locomotor agility was conducted on PND 45, 49, and 53. The mouse was placed on a 9.5 cm section of PVC rod measuring 3.81 cm in diameter surrounded by plastic walls and elevated 52 cm from the floor to prevent climbing or jumping off the rod. The animal received five trials on 3 separate testing days, each separated by 4 days to minimize motor learning. For the first trial, the mouse was placed on a stationary rod and the time the animal remained on the rod up to a maximum time of 60 sec was recorded. On the second and third trials, the mouse was placed on a continuously rotating rod at a constant rotation speed of 2.5 rotations per min and the

time the mouse was able to remain on the rod up to a maximum time of 60 sec was recorded. On the fourth and fifth trials, the mouse was placed on a rod rotating at an accelerating rate from 2.5 rotations per min up to 10.5 rotations per min and the time the mouse remained balanced on the rod up to a maximum time of 180 sec was recorded. The entire apparatus was cleaned with 75% ethanol solution between each trial.

***Elevated plus maze*.** Anxiety-like behaviors based on the rodent innate preference for enclosed, dark spaces was assessed in the EPM for 3 consecutive days on PND 54 – 56. One 5 min trial was conducted on each of 3 consecutive days wherein each mouse was placed on a black reflective Plexiglas surface measuring 5 x 5 cm and elevated 63 cm above the floor. Four arms (35 cm long, 5 cm wide) extended from a central area at 90° angles. Two opposing arms were closed with surrounding 15 cm high walls and the other two opposing arms remained open with a surrounding 1 mm high edge. The four arms and the center surface were each surrounded by photo beam pairs with hardware connected to a PC computer. The Motor Monitor software (Kinder Scientific, LLC, Poway, CA) quantified the beam breaks as duration, distance traveled, entries, and time at rest in each zone within the apparatus (closed arms, open arms and center area). The entire apparatus was dimly lit with two 13-Watt black-light household CFL light bulbs (Feit Electric EcoBulb) with no overhead lighting. The time spent, distance traveled and entries recorded in the open arm along with the percent of the total of each variable (time, distance, and entries) recorded in the open arms were used in analyses. The percent of the total of each variable was calculated with the equation [open arms / (open arms + closed arms)] * 100 to account for possible differences in activity levels during testing. The entire apparatus was cleaned with 75% ethanol solution between each mouse.

All mice were re-tested in the EPM on PND 144 – 146 for evaluation of changes in anxiety-like behaviors from adolescence to adulthood. All testing conditions remained the same as those used initially.

***Social approach*.** Social behaviors were assessed on PND 60. The protocol quantified sociability and preference for social novelty, and was adapted from methods previously described (Lazar, Neufeld, & Cain, 2011; Moy et al., 2004; Nadler et al., 2004; Naert, Callaerts-Vegh, & D'Hooge, 2011; Silverman, Yang, Lord, & Crawley, 2010). Sociability was defined here as a tendency to initiate social contact. Preference for social novelty was defined as initiating social contact with a novel conspecific as compared to a conspecific from a previous interaction.

Apparatus. The social approach testing apparatus was a rectangular Plexiglas box divided into three separate chambers each measuring 19.5 cm x 39 cm x 22 cm including Plexiglas dividing walls with rectangular openings measuring 5 cm x 8 cm to allow for movement between chambers, which could be shut off by sliding down Plexiglas doors. A small stainless steel conspecific cage (Galaxy Pencil/Utility Cup, Spectrum Diversified Designs, Inc, Streetsboro, OH), measuring 10 cm in height and 10 cm in diameter at its base, was placed in each outer chamber, and had vertical bars that allowed minimal contact while preventing fighting. A digital video camera connected to a PC computer with the software program ANY-maze (Stoelting Co., Wood Dale, IL.) tracked the behavior of the mouse within the apparatus and time spent in each investigation zone surrounding the conspecific cages. These data were used as an index of social contact, entries into the outer chambers as an index of general exploration, and total distance traveled as an index of general activity levels. The investigation zones encompassed an

area of 2 cm around the conspecific cages. An entry into the chambers was recorded when 80% of the mouse's body was inside the chamber, whereas only the head was tracked in the investigation zone to quantify intention to investigate the conspecific. The testing room was lit with two 500-watt compact halogen lights (Coleman) placed on the floor with no overhead lighting. The entire apparatus was cleaned with Nolvasan solution. The conspecific cages were cleaned with 75% ethanol solution between each mouse.

Trials. The social approach task consisted of four, consecutive 10 min trials. For the first trial, the mouse was placed in the middle chamber with the doors to the outer chambers shut and allowed 10 min to habituate to the apparatus. During the second trial (habituation trial), the mouse was allowed to freely investigate and habituate to all three chambers, including the empty conspecific cages, for 10 min. Performance of the mouse during the third trial (sociability trial) allowed for the evaluation of sociability to an unfamiliar, gender-matched conspecific (C57BL6) placed in one conspecific cage versus an empty conspecific cage. Again, the mouse was allowed to move freely within the apparatus for 10 min. During the fourth trial (preference for social novelty trial), the now familiar conspecific remained in the apparatus, and a new, unfamiliar gender-matched conspecific (C57BL6) was placed in the other conspecific cage. The mouse was allowed to move freely within the apparatus for 10 min, and the mouse's preference for social novelty was quantified. Outer chamber placement of conspecifics for the sociability trial was counterbalanced between exposure groups and placement of familiar and novel conspecifics for the preference for social novelty trial was randomized.

All mice were tested again in the social approach paradigm beginning on PND

178 for evaluation of long-term changes in social behaviors further into adulthood. All conditions during re-testing remained the same those used initially.

Conditioned fear. All mice were evaluated in the conditioned fear paradigm on PND 194 – 196 as described in previous studies (Khuchua et al., 2003; Wozniak, Xiao, Xu, Yamada, & Ornitz, 2007). Briefly, each mouse was habituated to and tested in a Plexiglas chamber, which measured 26 cm x 18 cm high x 18 cm and contained a metal grid floor, an LED light bulb and an inaccessible peppermint odorant. The chamber light turned on at the start of each trial and remained illuminated. The testing session on day 1 was 5 min during which time an 80 dB tone (white noise) sounded for 20 sec at 100 sec, 160 sec and 220 sec. A 1.0 mA shock (unconditioned stimulus; UCS) was paired with the last two sec of the tone (now conditioned stimulus; CS). The baseline freezing behavior during the first two min and the freezing behavior (conditioned response; CR) during the last 3 min was quantified through the computerized image analysis software program FreezeFrame (Actimetrics, Evanston, IL). This measure allowed for simultaneous visualization of behavior while adjusting a "freezing threshold," which categorized behavior as freezing or not freezing during 0.75 sec intervals. Freezing was defined as no movement except for normal respiration, and the data were presented as percent of time spent freezing. The testing session on day 2 was for 8 min. The light was illuminated during the entire trial and no tones or shocks were presented. This procedure allowed for the evaluation of freezing behavior (CR) in response to the contextual cues associated with the shock stimulus (UCS) from day 1. The testing session on day 3 was 10 min in duration and the context of the chamber was changed to an opaque Plexiglas-walled chamber containing a different (coconut) odorant. The 80 dB tone (CS) began at 120 sec

and lasted the remainder of the trial. Freezing behavior to habituation to the new context (pre-CS) was quantified during the first two min. Freezing behavior (CR) to the auditory cue (CS) associated with the shock stimulus (UCS) from day 1 was quantified during the remaining 8 min. Shock sensitivity was evaluated following testing as previously described (Khuchua et al., 2003).

Statistical Analyses

All statistical analyses were performed using the SPSS statistical program for PC computers, version 18. Means and standard errors were computed for each measure. Analyses involved factorial ANOVAs, including repeated measures ANOVAs (rmANOVAs) where appropriate, and one-way ANOVAs. The appropriate correction, Huynh-Feldt or Greenhouse-Geisser, was applied to violations of the sphericity assumption within a rmANOVA. With a statistically significant *F* value, the Tukey's HSD method was used as a post hoc test. With a statistically significant interaction between main factors on the factorial ANOVAs, simple main effects were calculated. Results provided between group and within group differences. Multiple comparisons were Bonferroni adjusted. Probability value for all analyses was $p < .05$.

Results

Body Weights

A 2 x 2 ANOVA was conducted on body weights on PND 3 with exposure and gender as the main factors to illuminate any differences in initial body weights that may confound other body weight analyses. The analysis yielded no significant effects

involving exposure or gender indicating no differences in body weights prior to treatment.

A 2 x 2 x 5 rmANOVA was performed to analyze body weights during the postnatal treatment phase with exposure and gender as the main factors and age (PND 4 – 8) as the within-subjects repeated measure. A statistically significant three-way exposure x gender x age interaction was further analyzed for simple main effects, $F(1.754, 54.367)=3,457$, $p=.044$, revealing female ISO mice weighed less than female controls on PND 8, $F(1, 31)=6.111$, $p=.019$ (Figure 10). A statistically significant two-way exposure x age interaction, $F(1.754, 54.367)=3.579$, $p=.040$, was further analyzed for simple main effects revealing ISO mice weighed less than controls on PND 8, which is likely due to the differences in female body weights as demonstrated by the three-way interaction, $F(1, 31)=5.882$, $p=.021$.

A 2 x 2 x 6 rmANOVA was conducted to examine post-treatment body weights with exposure and gender as the main factors and age (PND 9 – 14) as the within-subjects repeated measure. No statistically significant effects involving exposure were revealed.

A 2 x 2 x 7 rmANOVA was performed to examine differences in pre-weaning body weights with exposure and gender as the main factors with age (PND 15 – 21) as the within-subjects repeated measure. No statistically significant effects involving exposure were revealed.

A 2 x 2 x 4 rmANOVA was conducted to analyze post-weaning body weights with exposure and gender as the main factors and age (PND 30, 38, 45, and 60) as the within-subjects repeated measure. No statistically significant effects involving exposure were detected.

To summarize, body weights are a marker of general health. The only significant difference occurred on PND 8 between female mice such that female ISO-treated mice weighed less than female controls. Overall, ISO did not adversely influence body weights.

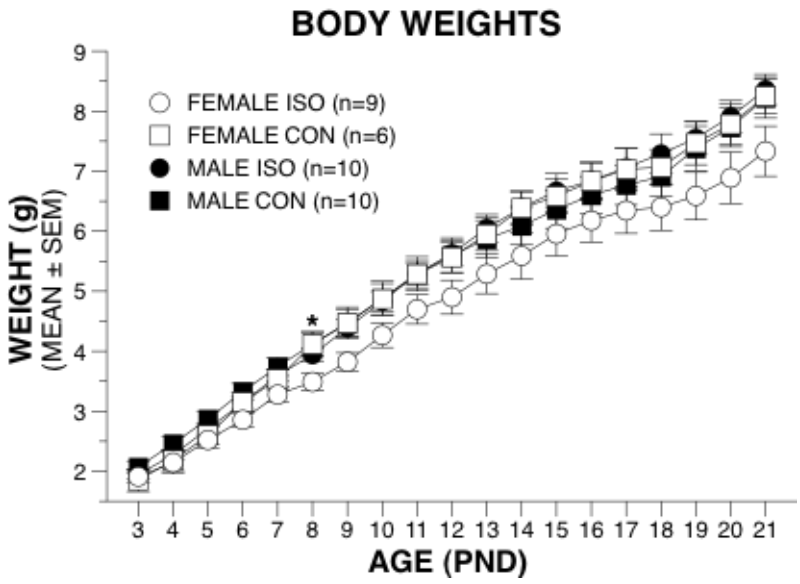

Figure 10. Female mice exposed to 1.5% ISO for 3 hr on PND 3+5+7 weighed less than female controls on PND 8, which immediately followed the final treatment day. * Denotes a statistically significant difference between female exposure groups, p=.019.

Reactivity to Handling, Sensorimotor Responses, and Morris Water Maze Performance

Mice exposed to ISO or room air on PND 3+5+7 were comparably reactive to handling. There were no significant differences between exposure groups across 3 assessment days revealed by a 2 x 2 x 3 rmANOVA with exposure and gender as the main factors and assessment day (1 – 3) as the within-subjects repeated measure. All mice exhibited a decrease in reactivity to handling across days, $F(2, 62)=8.237$, p=.001.

An ANOVA with exposure and gender as between-groups factors was performed on all sensorimotor variables. No significant differences were found among mice exposed

to ISO in strength, balance, and coordination performance compared with the control mice.

A rmANOVA was performed on the latency, path length, and swimming speed data from both the cued and place trials with exposure and gender as the main factors and blocks of trials (1 – 4 and 1 – 5, respectively) as the repeated, within-subjects measure. No significant effects of exposure were revealed for either cued or place trial data. All mice exhibited improved performance across time during cued trials, as evinced by a significant main effect of blocks of trials for latency and path length scores, $F(2.635, 81.672)=29.064$, $p=.000$ and $F(2.593, 80.379)=25.454$, $p=.000$, respectively. All mice exhibited improved performance across time during place trials, as indicated by a significant main effect of blocks of trials for latency and path length scores, $F(3.683, 114.187)=8.493$, $p=.000$ and $F(3.367, 104.369)=12.152$, $p=.000$. No differences were observed in swimming speeds for either cued or place trials.

The ISO mice demonstrated retention comparable to the control mice for the platform quadrant and location during the probe trial. A separate 2 x 2 ANOVA was conducted on target quadrant time and platform crossings with exposure and gender as the main factors. No statistically significant differences were found. A 2 x 2 x 4 rmANOVA with time in each of the four quadrants as the repeated measure and exposure and gender as the main factors revealed spatial biases within the ISO mice, $F(3, 29)=76.760$, $p=.000$, and control mice, $F(3, 29)=36.219$, $p=.000$, indicating more time spent by both groups in the target quadrant compared to the left, right and opposite quadrants, all $p=.000$.

In summary, no disruptions were observed among ISO-treated mice in reactivity to handling responses, sensorimotor skills, or MWM performance.

Locomotor Activity

Factorial ANOVAs with the main factors exposure and gender were conducted on all outcome variables: total ambulations, rearing, time at rest, time in center area, distanced traveled in center area, entries into center area, and distance traveled in peripheral area. No significant effects involving exposure or gender were revealed.

During exploratory analyses, exposure differences within the males were illuminated which guided the decision for re-testing certain paradigms. Therefore, the within-gender post hoc analyses are reported where appropriate. A post hoc one-way ANOVA with the main factor exposure was conducted separately for males and for females. Male ISO mice exhibited significantly less total ambulations, traveled significantly less distance within the peripheral area of the enclosure, and spent more time at rest compared with male controls, $F(1, 18)= 5.148 – 5.485$, $p<.037$ (Figure 11A-C). No significant effects involving exposure were revealed within the female mice.

In order to assess habituation to the testing enclosure and exploratory behaviors over time, the total ambulations and rearing data for the 1 hr session was divided into six 10-min blocks. A 2 x 2 x 6 rmANOVA conducted on total ambulations across six 10-min blocks with exposure and gender as main factors yielded comparable ambulatory activity among all mice. The analysis revealed a significant decrease over time indicating, on the average, habituation to the testing enclosure, $F(4.764, 147.697)=34.363$, $p=.000$.

Separate post hoc 2 x 6 rmANOVAs were conducted for each gender. No significant effects involving exposure were revealed within the female mice, and all

comparably habituated to the testing enclosure, $F(5, 65)=12.221$, $p=.000$. Male ISO mice exhibited less ambulatory activity across all six 10 minute blocks compared with male controls, $F(1, 18)=5.485$, $p=.031$ (Figure 11D). All male mice habituated to the testing enclosure, $F(4.016, 72.293)=22.003$, $p=.000$.

A 2 x 2 x 6 rmANOVA conducted on rearings across six 10-min blocks with exposure and gender as main factors revealed, in general, all mice comparably decreased exploratory behaviors over time, $F(5, 155)=31.934$, $p=.000$. Separate post hoc 2 x 6 rmANOVAs were conducted for each gender. No significant effects involving exposure were found for either males or females.

All mice were re-tested in the 1 hr locomotor activity chambers on PND 137 to investigate any chronic differences between ISO and control mice. Factorial ANOVAs were performed on all variables with exposure and gender as the main factors. No significant effects involving exposure or gender were revealed for any outcome variable. Separate analyses were conducted within each gender for comparison with the initial analyses. No significant effects involving exposure were revealed for males or females.

A 2 x 2 x 6 rmANOVA conducted on total ambulations across six 10-min blocks with exposure and gender as main factors yielded a statistically significant three-way interaction among blocks of min, exposure and gender, $F(5, 155)=2.626$, $p=.026$ (Figure 11E). Further analysis for simple main effects revealed female ISO mice exhibited more total ambulations compared to female controls during block 1 (min 0-10) and block 2 (min 11-20), $F(1, 31)=4.520 – 6.807$, $p<.043$. Female ISO mice also exhibited more total ambulations compared to male ISO mice during block 1 (min 0-10), $F(1, 31)=6.002$, $p=.020$. A statistically reliable main effect of blocks of minutes, $F(5, 155)=53.759$,

p=.000, revealed the mice habituated to the testing environment over blocks of minutes. Separate 2 x 6 rmANOVAs were conducted within each gender. A statistically significant interaction effect between exposure and blocks of min was further analyzed for simple main effects, $F(5, 65)=2.428$, $p=.044$. Female ISO mice exhibited more total ambulations during the first three 10-min blocks, $F(1, 13)=4.965 – 7.537$, $p<.045$. No similar effects in males were statistically reliable.

A 2 x 2 x 6 rmANOVA conducted on rearings across six 10-min blocks with exposure and gender as main factors revealed all mice decreased exploratory behaviors over time, $F(4.579, 141.952)=7.272$, $p=.000$. No significant effects involving exposure were revealed. Separate 2 x 6 rmANOVAs with each gender revealed no significant effects involving exposure for either gender.

In summary, the juvenile activity measures revealed statistically significant differences between ISO mice and controls, but only in males. During adulthood, slight disruptions in activity levels were detected in female ISO mice.

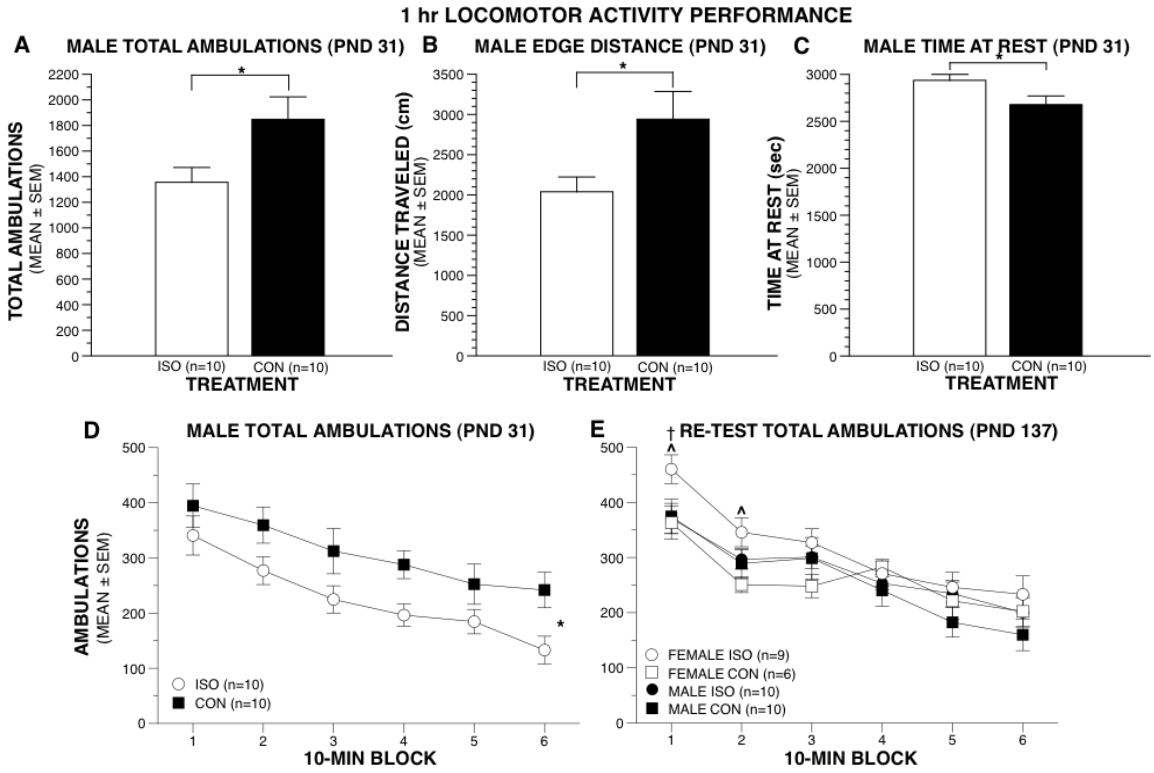

Figure 11. Exposure to 1.5% ISO for 3 hr on PND 3+5+7 disrupted activity levels in male mice when tested in the 1 hr locomotor activity test on PND 31 and in female mice when tested in the 1 hr locomotor activity test on PND 137. (A) Male ISO mice exhibiting less total ambulations, (B) traveled less distance in the edge of the enclosure, and (C) spent more time at rest compared to male control mice. (D) Male ISO mice exhibited decreased total ambulations compared to male controls over six 10-min blocks during 1 hr locomotor activity testing on PND 31. (E) Female ISO mice displayed more total ambulation compared to female control mice and male ISO mice during the beginning of re-testing of in the 1 hr locomotor activity test on PND 137. ***** Denotes a statistically significant difference between male exposure groups $p<037$. **^** Denotes a statistically significant difference between female exposure groups, $p<.044$. **†** Denotes a statistically significant difference between male and female ISO mice, $p=.020$.

Rotarod

No statistically significant effects involving exposure or gender were revealed by a 2 x 2 x 3 rmANOVA performed on stationary rotarod time with exposure and gender as the between-subjects factors and trial (1 – 3) as the repeated measure. A 2 x 2 x 2 x 3 rmANOVA was performed on continuous rotarod time with exposure and gender as the between-subjects factors and test day (1 – 3) and trial (1 and 2) as the within-subjects repeated measures. No statistically significant main effects involving exposure or gender were revealed.

A 2 x 2 x 2 x 3 rmANOVA was performed on time spent on the accelerating rotarod with exposure and gender as the between-subjects factor and test days (1 – 3) and trials (1 and 2) as the within-subjects repeated measures. A statistically significant interaction between test day and exposure, $F(2, 62)=3.153$, $p=.049$, was further analyzed for simple main effects (Figure 12). Compared to control mice, ISO mice spent significantly less time on the accelerating rotarod on test day 3, $F(1, 31)=5.123$, $p=.031$.

To summarize, by the end of testing, ISO mice did not remain on the accelerating rotarod as long as the control mice suggesting disruptions in fine motor coordination among ISO-treated mice.

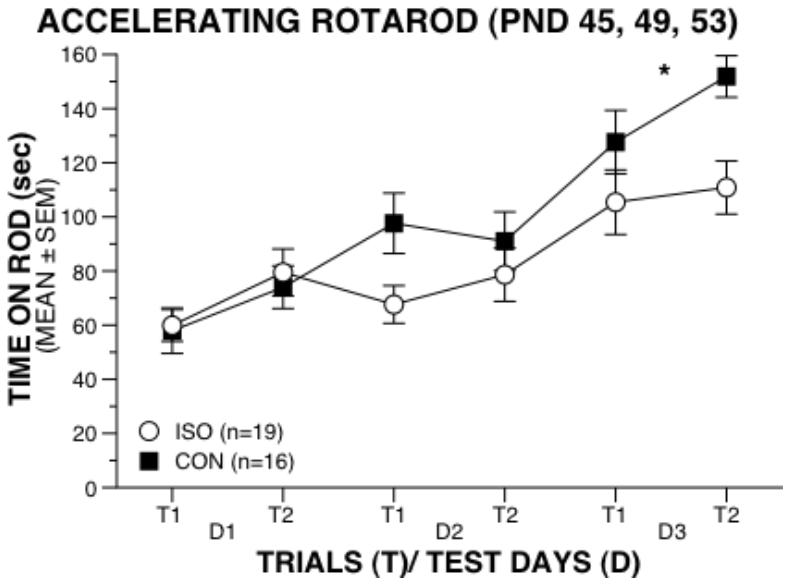

Figure 12. ISO mice remained on the accelerating rotarod for less time compared to control mice on day 3. ***** Denotes a statistically significant difference between exposure groups, $p=.031$.

Elevated Plus Maze

A 2 x 2 x 3 rmANOVA was performed on each variable (time, distance, and entries) recorded in the open arms with exposure and gender as the main factors and test day (1 – 3) as the repeated measure. No significant effects involving exposure or gender were revealed for time or distance in the open arms. Analysis of open arm entries yielded

a statistically significant gender x test day interaction, $F(2, 62)=5.718$, $p=.005$. Further analysis for simple main effects revealed male mice made fewer entries into the open arms compared to female mice on test day 1, $F(1, 31)=8.858$, $p=.006$.

The significant interaction between gender and test day allowed for post hoc comparisons within males and females separately. Factorial 2 x 3 rmANOVAs were performed for entries into the open arms males and females separately with exposure as the main factor and test day (1 – 3) as the repeated measure. No significant effects involving exposure was revealed for males or females.

A 2 x 2 x 3 rmANOVA was performed on the percent of the total of each variable (time, distance, and entries) recorded in the open arms with exposure and gender as the main factors and test day (1 – 3) as the repeated measure. No significant effects involving exposure or gender were revealed for percent total distance or total entries in the open arms. Analysis of percent total time yielded a statistically significant gender x test day interaction, $F(1.945, 60.283)=3.381$, $p=.038$. Further analysis for simple main effects revealed male mice spent less percent of total time in the open arms compared to female mice on test day 1, $F(1, 31)=5.242$, $p=.029$.

The significant interaction between gender and test day allowed for post hoc comparisons within males and females separately, therefore, 2 x 3 rmANOVAs were performed for percent of total time in the open arms for males and females separately with exposure as the main factor and test day (1 – 3) as the repeated measure. No significant effects involving exposure were revealed within the female mice. Within the male mice, a significant main effect of exposure was revealed indicating male ISO mice

spent less percent of total time in the open arms, $F(1, 18)=5.425$, $p=.032$, relative to male controls (Figure 13).

All mice were re-tested in the EPM on PND 144 – 146 in order to evaluate chronic differences on EPM performance. No significant effects involving exposure were revealed for any outcome variables. Post hoc 2 x 3 rmANOVAs were performed with exposure as the main factor and test day (1 – 3) as the repeated measure for males and females separately on entries into the open arms and percent of total time spent in the open arms to replicate the analyses from the initial testing session. No significant effects involving exposure were detected within males or females for either variable.

To summarize, adolescent male mice exposed to ISO postnatally demonstrated slightly heightened anxiety levels compared to adolescent male controls by spending less percent of time in the open arms of the EPM. These differences did not continue into adulthood.

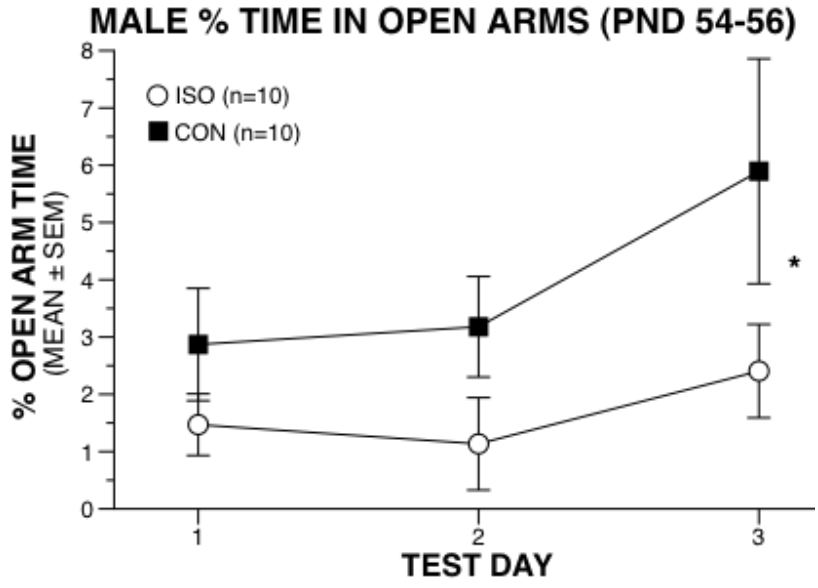

Figure 13. Male ISO spent less percent of total time in the open arms compared to male controls during elevated plus maze testing on PND 54-56. * Denotes a statistically significant difference between male exposure groups, $p=032$.

Social Approach

Factorial 2 x 2 x 2 rmANOVAs were performed on time spent in the investigation zone with exposure and gender as the main factors and investigation zone as the repeated measure for each social approach trial: habituation, sociability, and preference for social novelty. To illuminate preferences for either investigation zone within each exposure group, pairwise comparisons of the investigation zones were performed within ISO and control mice separately. A 2 x 2 x 3 rmANOVA was performed on number of entries into the chambers with exposure and gender as the main factors and chamber (middle vs. empty vs. novel) as the repeated measure for each trial to assess differences in general exploration during social approach testing. A 2 x 2 ANOVA was conducted on total distance traveled to illuminate any differences in general activity during testing.

Habituation. Neither ISO mice nor control mice demonstrated investigation zone biases during the habituation trial, presenting no confounding issues for the interpretation of the results from the sociability or preference for social novelty trials. No significant effects involving exposure or gender were detected for chamber entries or total distance traveled.

Sociability. In general, the mice demonstrated a clear preference to investigate the novel conspecific instead of the empty cage by spending more time in the novel conspecific investigation zone (novel zone), $F(1, 31)=117.333$, $p=.000$. Pairwise comparisons revealed the preference to spend time in the novel zone for both the ISO and control mice, $F(1, 31)= 51.581 – 67.629$, $p=.000$. No significant effects involving exposure or gender were revealed for chamber entries or total distance traveled.

Preference for social novelty. The analysis yielded a significant effect of zone for time spent in the investigation zone, $F(1, 31)=23.628$, $p=.000$, illustrating a general preference of the mice to spent time investigating the novel conspecific rather than the familiar conspecific. The preference to investigate the novel conspecific was shared by both the ISO and control mice, $F(1, 31)=5.662 – 19.266$, $p<.024$. A significant zone by gender interaction, $F(1, 31)=5.545$, $p=.025$, was further analyzed for simple main effects. Male mice spent more time in the novel zone compared to female mice, $F(1, 31)=10.040$, $p=.003$ (Figure 14). Overall, male mice spent more time in the novel zone compared to the familiar conspecific investigation zone (familiar zone), $F(1, 31)=31.095$, $p=.000$. Female mice did not demonstrate this preference. A significant main effect of gender revealed male mice overall spent more time than females investigating either investigation zone, $F(1, 31)=5.032$, $p=.032$.

Further examination of the influence of ISO on preference for social novelty within each gender was conducted due to the significant interaction between zone and gender and the main effect of gender. A 2 x 2 rmANOVA was performed on time spent in each investigation zone with exposure as the main factor for male and female mice separately. Male mice demonstrated a preference to spend time in the novel zone, $F(1, 18)=24.940$, $p=.000$ (Figure 14). This was shared by ISO and control males, $F(1, 18)=8.031 – 21.542$, $p<.012$. In general, female mice did not demonstrate a preference. Female controls exhibited a trend towards spending more time investigating the novel conspecific, $F(1, 13)=3.339$, $p=.078$. The non-significance among the female controls is likely due to a lack of power given the sample size of six for the female control group.

No significant effects involving exposure or gender were revealed for chamber entries or total distance traveled.

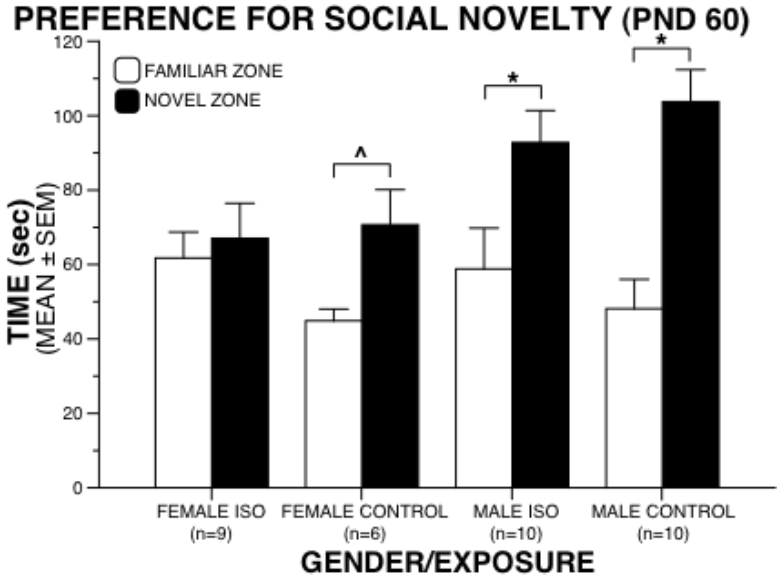

Figure 14. Male mice, but not female mice, demonstrated a preference to spend time in the investigation zone surrounding the novel conspecific compared to that surrounding the familiar conspecific during the preference for social novelty trial of the social approach test on PND 60. * Denotes a statistically significant difference between investigation zones, $p<.012$. ^ Denotes the difference between investigation zones approached significant, $p=.078$.

Social Approach Re-test

Habituation. Neither ISO mice nor control mice demonstrated investigation zone biases during the habituation trial, presenting no confounding issues for the interpretation of the results of the sociability or preference for social novelty trials. Female mice spent more time in the investigation zones overall than did male mice, $F(1, 31)=11.589$, $p=.002$. No significant effects involving exposure or gender were revealed for chamber entries. A significant main effect of gender, $F(1, 31)=5.478$, $p=.026$, for total distance traveled revealed, on the average, female mice traveled a greater total distance compared to male mice. No significant effects involving exposure were revealed for total distance traveled.

Sociability. During the sociability trial, on average the mice preferred to investigate the novel conspecific mouse by spending more time in the novel zone compared to the investigation zone surrounding the empty cage, $F(1, 31)=132.779$, $p=.000$, regardless of ISO or room air exposure, $F(1, 31)= 56.521 – 78.950$, $p=.000$. No significant effects involving exposure or gender were revealed for chamber entries or total distance traveled.

Preference for social novelty. An overall preference to spend more time in the novel zone compared to the familiar zone was demonstrated by the mice as a whole, $F(1, 31)=37.798$, $p=.000$. This preference was shared by both the ISO and control mice, $F(1, 31)= 12.243 – 28.265$, $p<.002$.

To replicate analyses from initial testing, separate rmANOVAs were performed on time spent in the investigation zones for males and females. In general, both male and female mice demonstrated a preference to spend time investigating the novel mouse, $F(1, 18)=46.900$, $p=.000$ and $F(1, 13)=8.244$, $p=.013$, respectively. Male ISO and male control mice exhibited a preference to investigate the novel mouse by spending more time in the novel zone, $F(1, 18)= 16.348 – 31.830$, $p<.002$. Only the female ISO mice demonstrated a preference to spend more time in the novel zone, $F(1, 13)=6,585$, $p=.023$ (Figure 15). Female control mice did not display a preference to investigate the novel conspecific, $F(1, 13)=2.597$, $p=.131$. This lack of a significant effect within the female control mice is likely due to a lack of power given the sample size of six in this group, and the lack of significant gender effects in the main analysis. No significant effects involving exposure or gender were detected for chamber entries or total distance traveled.

To summarize the social approach findings, female mice exposed postnatally to ISO failed to demonstrate a preference for social novelty during early adulthood, which did not remain into later adulthood.

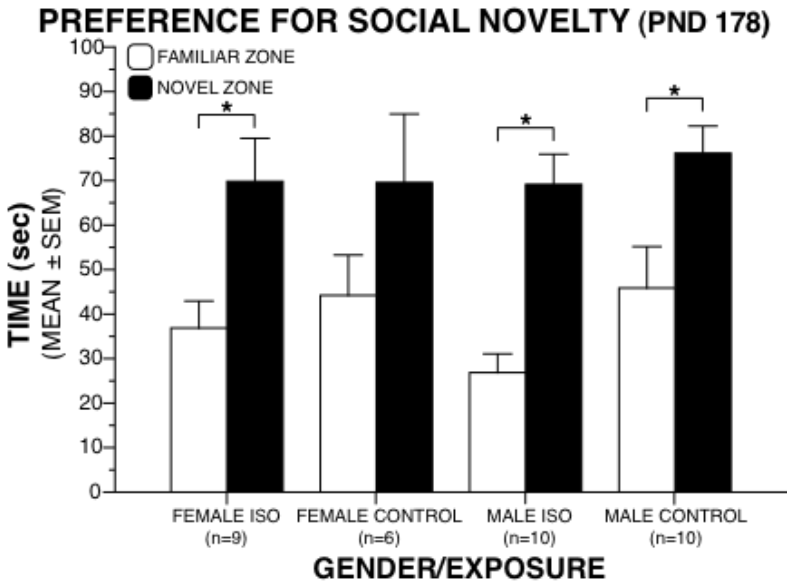

Figure 15. All groups demonstrated a preference to spend time in the investigation zone surrounding the novel conspecific compared to that surrounding the familiar conspecific, except for the female control mice, during the preference for social novelty trial of the social approach re-test on PND 178. This lack of a difference within female controls is likely due to the small number of mice in this group. ***** Denotes a statistically significant difference between investigation zones, $p<024$.

Conditioned Fear

To examine baseline-freezing behavior prior to the pairing of tone and shock on day 1, a rmANOVA was performed on percent time spent freezing with main factors of exposure and gender and a within-subject repeated-measure of min (1 vs. 2). All mice exhibited comparable baseline freezing behavior during min 1 and 2 of test day 1 (Figure 16).

A rmANOVA was performed on percent time spent freezing during the pairing of tone and shock on day 1, with exposure and gender as the main factors and min (3 – 5) as the within-subjects repeated measure. A significant exposure x min interaction during the pairing of tone and shock on test day 1, $F(1.789, 55.467)=6.140$, $p=.005$, was further

analyzed for simple main effects (Figure 16). ISO mice froze less during the min 3 compared to controls, $F(1, 31)=5.403$, $p=.027$. A significant main effect of min revealed the percent time spent freezing increased, in general, across min following each pairing of shock with tone, $F(1.789, 55.467)=75.668$, $p=.000$. The significant main effect of gender, $F(1, 31)=4.909$, $p=.034$, reveals male mice froze a greater percent of time compared to female mice.

The influence of ISO on the percent time spent freezing during the pairing of tone and shock was further examined within males and females separately due to the main effect of gender in the main analysis. No significant effects involving exposure were revealed within the female mice. The analysis of male mice freezing behavior yielded a min x exposure interaction, $F(2, 36)=6.083$, $p=.005$, similar to the main analysis. Male ISO mice exhibited less freezing behavior compared to male controls during min 3, $F(1, 18)=6.606$, $p=.019$.

A rmANOVA with exposure and gender as the main factors and min (1 – 8) was conducted on percent time spent freezing during contextual fear conditioning testing on day 2. No significant effects involving exposure or gender were revealed. To examine the influence of the differences in gender observed during day 1 pairing of tone and shock, separate analyses within males and females were performed. No significant effects involving exposure were revealed within the male mice (Figure 17A). Comparisons of female exposure groups across time revealed female controls exhibited more freezing behavior compared to female ISO mice during min 3, 4, and 8, $F(1, 13)=6.111 – 9.172$, $p<.029$ (Figure 17B).

To analyze baseline freezing behavior in response to an altered environment, a rmANOVA with exposure and gender as the main factors and min (1 – 2) during test day 3 served as the within-subjects repeated measure. No significant effects involving exposure or gender were revealed. Separate analyses within males and females once again were conducted, yet no significant effects involving exposure were revealed within either analysis.

Freezing behaviors during the auditory cue fear conditioning testing on day 3 was examined by performing a rmANOVA with exposure and gender as the main factors and min (3 – 10) as the within-subjects repeated measure. No significant effects involving exposure or gender were revealed (Figure 18). Separate analyses within males and females yielded no significant main effects involving exposure.

A 2 x 2 ANOVA was performed on shock sensitivity variables, flinch, vocalization, and run, with exposure and gender serving as factors. A significant two-way interaction between exposure and gender was revealed for flinch and vocalization, $F(1, 31)= 6.643 – 8.937$, $p<.016$. Analysis for simple main effects of flinch revealed male ISO mice required a higher current of shock to flinch compared to male control mice and female ISO mice, $F(1, 31)=7.980 – 9.568$, $p<.009$. The same pattern was revealed by analysis for simple main effects of vocalization: male ISO mice required a higher current of shock to vocalize compared to male control mice and female ISO mice, $F(1, 31)= 5.237 – 13.539$, $p<.030$. A main effect of gender was revealed for vocal and run, $F(1, 31)=5.356 – 11.826$, $p<.028$, indicating male mice required a higher current of shock to vocalize or run compared to females.

Repeated measures ANCOVAs were performed on percent time spent freezing on each test day with exposure and gender as the main factors, min as the within-subjects repeated measure and flinch, vocalization, or run serving as the covariate. A significant covariate was not detected by any analysis, indicating shock sensitivity, as measured by flinch, vocalization or run, was not related to the freezing behavior of the mice.

In summary, conditioned fear testing allows for comparisons of hippocampal- and amygdala-involved associative learning. Female ISO mice exhibited impaired contextually cued fear conditioning.

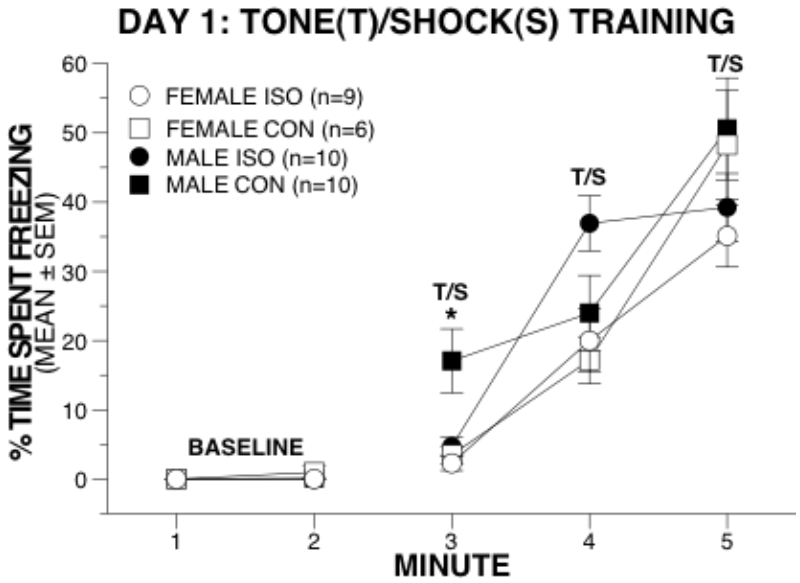

Figure 16. Male ISO mice exhibited less freezing behavior compared to male controls during min 3, directly following the first pairing of tone and shock, during fear conditioning on PND 194. ***** Denotes a statistically significant difference between male exposure groups, *p*=.019.

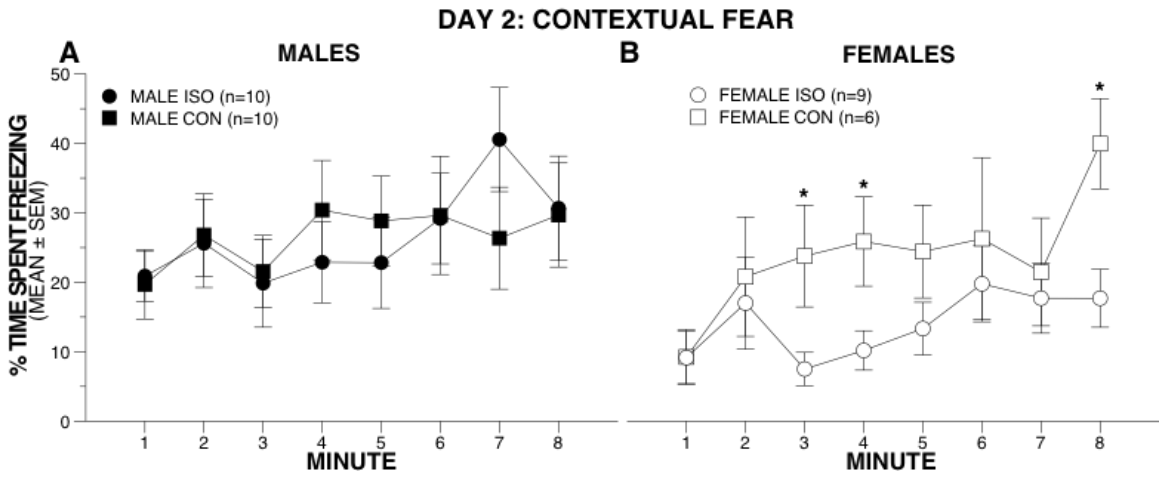

Figure 17. Differences in freezing behavior were observed only within female mice during the contextual-cue fear-conditioning test on PND 195. (A) No differences were observed between male exposure groups. (B) Female ISO mice spent less percent of time freezing compared to female controls during min 3, 4, and 8. ***** Denotes a statistically significant difference between female exposure groups, $p<.029$.

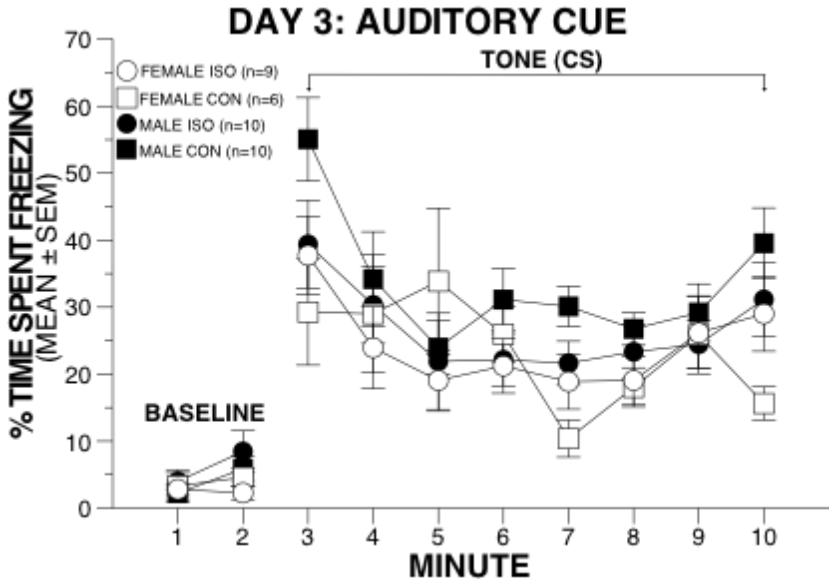

Figure 18. No differences were observed in freezing behavior between exposure groups during auditory-cue fear conditioning testing on PND 196.

Discussion

Experiment 2 was designed to investigate the influence of multiple postnatal ISO exposures on long-term function in the mouse. Male and female mice were exposed to 1.5% ISO for 3 hr on PND 3+5+7 and behaviorally tested beginning during the juvenile

stage and continuing through adulthood.

Postnatal ISO exposure decreased the body weight of the female mice on PND 8, the day following the third and final ISO exposure. This difference was not observed on subsequent days. Male body weights were not affected. Because this difference was transient, we do not believe malnutrition is a factor in any later functional differences observed between female exposure groups.

We hypothesized that the greatest behavioral disruption following postnatal ISO exposure would occur in the cognitive domain. This was not supported by MWM performance. The ISO and control mice exhibited comparable spatial acquisition and spatial memory retention during all trials of MWM. Disruptions were observed during conditioned fear testing, however. Female mice exposed to ISO displayed less freezing behavior compared to female controls during the contextual-cue portion of conditioned fear testing on PND 195. No differences in freezing behaviors among the groups were observed during the auditory-cue portion of conditioned fear testing.

We hypothesized that multiple neonatal ISO exposures would adversely influence both emotional and social behaviors. Mild functional disturbances were observed in both domains and were gender-dependent. Overall groups, adolescent male ISO mice were slightly more anxious as measured by reduced percent of total time in the open arms compared to male controls during EPM testing on PND 54-56. Differences were only observed for one variable of EPM performance: percent of time in the open arms. The lack of differences for the other five variables suggests the difference in percent time spent in the open arm may not represent true anxiety differences.

Anxiety did not extend into the social realm for the ISO males. During social

approach testing on PND 60, male ISO and control mice demonstrated a similar preference for social novelty by spending more time investigating a novel conspecific compared to a familiar conspecific. Female ISO mice, however, spent comparable time investigating both the novel and familiar conspecifics, demonstrating no preference for social novelty. Female controls demonstrated a trend towards this preference. The non-significant differences among female control mice were likely due to the small number of mice in this group.

Differences in emotional or social behaviors did not persist long-term. No differences were observed between males exposure groups in EPM performance when re-tested on PND 144-146. During social approach re-testing on PND 178, all male mice and female ISO mice spent more time investigating the novel conspecific. Again, the absence of significant differences among the female controls was likely due to the small numbers of mice in this group.

We hypothesized that postnatal ISO exposure would not influence later general neuromotor functioning, but this was not supported by the data. Male ISO mice demonstrated decreased total ambulations and distance traveled within the edge area, and increased time at rest compared to male controls during testing in the 1 hr locomotor activity test on PND 31, indicating decreased activity levels in juvenile males following postnatal ISO exposure. Female ISO mice increased total ambulations relative to female controls and to male ISO mice during initial re-testing of locomotor activity at PND 137, indicating hyperactivity in adult females postnatally exposed to ISO.

Although we did not observe balance, strength, and coordination differences on the sensorimotor battery, fine motor coordination disruptions were observed on the third

day accelerated rotarod testing on PND 53. ISO mice remained on the accelerating rotarod for less time compared to controls.

The results of this experiment reveal multiple postnatal exposures to ISO induced mild cognitive, socio-emotional, and neuromotor deficits in later life that were age- and gender-dependent (Figure 19).

Multiple postnatal ISO exposures:

- disrupted freezing behaviors in female mice during contextual-cue portion of conditioned fear testing on PND 195.
- increased anxiety-like behaviors in male mice during elevated plus maze testing on PND 54-56.
- impaired preference for social novelty performance in female mice in the social approach test on PND 60.
- decreased activity levels in juvenile males during the 1 hr locomotor activity test on PND 31.
- induced initial hyperactivity in female mice during re-testing in the 1 hr locomotor activity test on PND 137.
- disrupted fine motor coordination performance on the accelerating rotarod during day 3 of rotarod testing on PND 53.

Figure 19. Exposure to 1.5% ISO for 3 hr on PND 3+5+7 induced cognitive, socio-emotional, and neuromotor deficits later in life that were age- and gender-dependent.

GENERAL DISCUSSION

Two clinical studies have linked multiple exposures to anesthesia prior to the age of four with an increased risk of developing learning disabilities (LD) in children (Flick et al., 2011; Wilder et al., 2009). These studies report this risk increases with longer cumulative duration of anesthetic exposure. It has also been reported that surgery during a child's first 3 years of life more than doubles the likelihood of a developmental or behavioral disorder diagnosis later in life (DiMaggio et al., 2009). During the first few years of life, a child's brain is undergoing a phase of development commonly called the brain growth spurt period, which includes high levels of synaptogenesis (Dobbing & Sands, 1979; West, 1987). The mechanism by which general anesthetics work is to either antagonize the NMDA receptor or potentiate the $GABA_A$ receptor (Forman & Chin,

2008). Exposure to anesthetics during the brain growth spurt period in rodents can induce abnormal levels of neuroapoptosis, which has the potential to alter neural circuitry and produce a range of behavioral disruptions during later development (Ikonomidou et al., 2001).

Two experiments were conducted to determine the developmental effects of exposure to the general anesthetic isoflurane (ISO) on acute apoptotic neurodegeneration and long-term behavior. In the first experiment, mouse pups were exposed to 1.5% ISO for 3 hr on postnatal day (PND) 3, 5, 7, 3+5, 5+7 or 3+5+7. Neuroapoptosis was measured by the density of activated caspase-3 (AC3) profiles and an increase in the AC3-positive profile density indicated an increase in neuroapoptosis. Regardless of age at exposure, ISO increased neuroapoptosis beyond control levels. Multiple ISO exposures induced distinct patterns of neuroapoptosis. Extent of the observed insult varied between brain regions depending on age at exposure rather than simply the numbers of exposures.

The second experiment examined the behavioral influence of 1.5% ISO exposure for 3 hr on PND 3+5+7 in a longitudinal manner beginning in the juvenile stage and continuing through adulthood. Immediately following weaning, the reactivity of the mice to handling was evaluated during which a higher score indicated a greater reaction to experimenter handling. As juveniles, the mice were assessed in the 1 hr locomotor activity test, which measured differences in activity levels by quantifying vertical and horizontal movements, and in a battery of sensorimotor tests that measured strength, coordination and balance through timed trials. Morris water maze (MWM) was used to evaluate spatial reference memory during the juvenile stage. A longer latency and path length distance to find the escape platform indicated worse spatial memory acquisition

during the place trials of the MWM, whereas less platform crossings and time spent in the target quadrant indicated worse spatial memory retention. During adolescence, the mice were evaluated on the rotarod test, during which a shorter duration on the rotarod indicated worse fine motor coordination, and in the elevated plus maze (EPM), which quantifies less time, entries and distance in the open arms as a marker of heightened anxiety levels. The social behaviors of the mice were evaluated during early adulthood in the social approach test, in which more time spent investigating a novel conspecific relative to an empty cage or familiar conspecific indicated normal sociability and preference for social novelty. Later in adulthood, the mice were re-evaluated in the 1 hr locomotor activity test, EPM, and social approach test. Finally, the ability of the mice to create an association between an adverse unconditioned stimulus and a conditioned stimulus was measured in the conditioned fear paradigm during which decreased freezing behaviors indicated a lack of conditioning to the stimuli.

A sampling of the significant differences between experimental and control groups included that ISO exposure disrupted the activity levels of juvenile males and adult females during 1 hr locomotor activity testing. In addition, fine motor coordination on the rotarod was disrupted in all ISO groups during adolescence. Adolescent ISO-treated male mice displayed slightly heightened anxiety levels in the EPM and young adult ISO-treated females exhibited altered social preferences in the social approach paradigm. Cognitive disturbances were observed in adult female ISO-treated mice in the fear-conditioning paradigm.

The current study confirms ISO is apoptogenic in the developing brain of mice. Consistent with previous rodent studies, ISO exposure increased neuroapoptosis in the

cerebral cortex, caudate, dorsal hippocampus, and thalamus (Jevtovic-Todorovic et al., 2003; Johnson et al., 2008; Kodama et al., 2011). These brain regions are implicated in specific behaviors and they defined the behaviors tested in experiment two.

The networks involving the hippocampus are vital for memory processes and hippocampal-cortical connections are central in various cognitive functions. Beyond functioning as a relay station for sensory information, the thalamus appears to play a role in controlling hippocampal function, including those interactions important for episodic memory (Aggleton et al., 2010). The brain regions examined in the current experiments also operate in frontal-subcortical circuits involved in motor, cognitive and emotional functions (Tekin & Cummings, 2002). The present study included measures of sensorimotor function, social responsiveness, emotions, and, primarily, cognitive behaviors.

Experimental hypotheses for Experiment 1 were suggested by the pertinent literature. Histological evaluation of neonatal brains revealed support for the experimental hypotheses. ISO induced abnormal neuroapoptosis in all four brain regions examined, but only in PND 5 mice. The greatest insult observed in PND 5 mice occurred in the caudate. ISO exposure on PND 3 induced apoptotic insult in all brain regions except the caudate, and the greatest ISO-induced neuroapoptotic response at this age occurred in the thalamus. The proapoptotic effects of ISO in the hippocampus were greatest on PND 3 and PND 5.

Damage to the cortex was observed in singly exposed mice on PND 3, 5 or 7. Indeed, only the cortex was damaged in PND 7 animals. This observation is inconsistent with previous studies of the impact of developmental exposure to proapoptotic agents on

the cortex (Ikonomidou, Bittigau, et al., 2000; Ikonomidou et al., 1999). An important comparison was between PND 3 and PND 7 groups because those are the groups most often included in prior studies. Here, the differences between PND 3 and 7 were statistically unreliable. In the current experiment, we observed a significant increase only in the neuroapoptotic response in the cortex on PND 5 compared to PND 3.

A notable distinction between these previous studies and the current experiment is the agent investigated. Previous studies explored the neuroapoptotic response as a function of developmental age following exposure to NMDA-antagonists and ethanol. ISO targets the GABAergic pathways. A study of 1.5% ISO exposure for 6 hr also revealed greater neuroapoptosis induced in the cortex and most thalamic nuclei examined on PND 7 compared to PND 3 (Yon et al., 2005). Only in the anteromedial thalamic nucleus was the neuroapoptotic response to ISO greater on PND 3 than PND 7. Another difference between the current experiments and those listed above is the species studied. These studies investigated the apoptogenic response of the developing rat brain, not the mouse brain.

Of the four brain regions examined in the current experiment, the cortex was the most vulnerable to ISO insult. This observation is illustrated by increased neuroapoptosis relative to controls regardless of age at exposure. These findings correspond with those of previous studies. Cortical neuroapoptosis has been observed following ISO exposure in the rat and mouse at all ages PND 3, 5, 6, and 7 (Johnson et al., 2008; Kodama et al., 2011; Yon et al., 2005). The cerebral cortex had the largest mass of the regions examined in the current study, and it comprises many distinctly functioning lobes. It is likely that the critical period of proapoptotic sensitivity within the cerebral cortex is much longer

than other, smaller regions of the developing mouse brain.

The age at insult has long been thought to be critical in the generation of behavioral disruptions. The oft-cited Kennard Principle is that recovery of function is better the earlier the age of injury. By contrast, the more recent suggestions from lesion studies (Kolb & Gibb, 2007) are that behavioral impairments can be more devastating when cortical injuries occur earlier in the brain growth spurt period (PND 1 – 5) than later (PND 7 – 14). The suggestions may apply to apoptotic injury, as well. In the current study, we found greater neuroapoptotic responses were induced by a single ISO exposure on PND 3 and 5 relative to PND 7, suggesting the developing mouse brain is more sensitive to the proapoptogenic properties of ISO earlier in the brain growth spurt period.

Clinical evidence of an association between multiple, but not single, anesthesia exposures prior to the age of 4 in children and an increased risk of later LDs. Those reports suggested that preclinical studies investigate the acute neurodegenerative effects of multiple versus single anesthesia exposures (Flick et al., 2011; Wilder et al., 2009). The current study adds to that literature by comparing single and multiple exposures to anesthesia in neonatal mice. We found evidence both consistent and contrary to those reports. Consistent were the findings that a dual exposure on PND 5+7 was more damaging than a single exposure on PND 7. Contrary to the reports in humans, a single exposure on PND 5 was responsible for greater apoptosis than exposures on PND 3+5.

Complexities arise with the interpretation of neuroapoptotic densities following multiple ISO exposures across days. Cells induced to undergo apoptosis following the first day of ISO exposure are phagocytosed by 24 hours (Kerr et al., 1972). Therefore, AC3 staining no longer marks apoptosis after this time point. Because our ISO exposures

were 48 hr apart, the observed neuroapoptosis in the brains exposed to dual ISO exposures is only that induced by the second ISO exposure. All evidence of the neuroapoptosis induced by the first ISO exposure will have been phagocytosed prior to AC3 staining. However, the first exposure to ISO influences the neuroapoptosis induced by subsequent exposures. This is supported by the differences observed in neuroapoptotic densities between single, double, and triple ISO exposures in brains stained for AC3 at the same age.

It is not clear how preceding ISO exposure(s) affect the observation that less neuroapoptosis was found following dual exposures on PND 3+5 whereas more neuroapoptosis occurred on PND 5+7. The apoptosis induced by the first ISO exposure leaves less neurons for the second ISO exposure to damage. This is likely what is happening with the neuroapoptotic densities following ISO exposure on PND 5 versus PND 3+5. That more neuroapoptosis was observed following ISO on PND 5+7 relative to that on PND 7 could be a physiological artifact of AC3 labeling along with other developmental processes occurring at that time which could be affecting the brain's environment. These factors could have prevented the AC3 stain from labeling properly.

Exposure to ISO on PND 3+5+7 mildly disrupted functioning of mice on several behavioral paradigms in an age- and gender-dependent manner in the present experiments. Neonatal ISO exposure resulted in a decrease in activity levels and a slight increase in anxiety levels in male mice as juveniles and adolescents, respectively. These results do not clearly support a conclusion that postnatal ISO exposure increases anxiety levels of young male mice. The decreased activity in the 1 hr locomotor activity test was not accompanied by a decrease in time spent or distance traveled in the center area of the

apparatus compared to controls, thus suggesting comparable emotionality levels among male exposure groups. The performance of the ISO-treated males in the EPM was only marginally indicative of heightened anxiety given that differences were observed on only one of the six variables. By adulthood, the behaviors of the ISO-treated male mice were no different from those of the controls during re-testing in the 1 hr locomotor activity test and EPM. The non-significant findings could have resulted from the large amount of handling these mice received by the experimenter following initial testing. Repeated testing in the 1 hr locomotor activity test and the EPM also may have affected the behaviors of the male mice. Previous exposure may have influenced the reactivity of the mice to the testing environment.

Previous research on the influence of neonatal ISO exposure on adolescent rodent EPM performance is inconsistent. Despite ISO exposure levels likely to produce apoptosis, male rats exposed to ISO *in utero* and male mice exposed to ISO on PND 6 exhibited no disruptions in EPM performance in two separate studies (Kodama et al., 2011; Palanisamy et al., 2011). Yet another study found male rats exposed to ISO on the day of birth spent less time in the open arms of the EPM, consistent with the current experiment (Rothstein et al., 2008). Rothstein et al. (2008) also reported the same deficits in female ISO-treated rats, which were not observed in the current experiment. Based on the previous and current findings, more research is needed to confirm the influence of neonatal ISO exposure on anxiety-related performance in the EPM.

Female ISO-treated mice began to exhibit behaviors indicative of cognitive disruptions in early adulthood. During social approach testing in early adulthood, females exposed to ISO failed to demonstrate a preference for social novelty. This suggests the

female ISO mice had different social preferences or the female mice were not able to discriminate between the two conspecifics and to recognize the one that had not been encountered previously. This possibly results from a cognitive deficit induced by ISO in females, as the time spent in close proximity to the two conspecifics was not different from that observed in controls, indicating the female ISO mice were not avoiding social contact. Consistent with possible cognitive disruption, the female ISO mice displayed decreased freezing behaviors in contextual fear conditioning later in adulthood. This suggests that the female ISO mice did not condition to the context in which the aversive stimulus (shock) occurred as strongly as female controls or that they did not retain that conditioning over a 24 hr period.

The current findings replicate fear-conditioning impairments reported previously in both rats and mice following neonatal ISO exposure. Adult rats exposed to ISO on PND 7 demonstrated impaired freezing behaviors during contextual fear conditioning, but not auditory-cued fear conditioning (Stratmann, Sall, et al., 2009). Impairment was observed in both portions of fear conditioning during adolescence in male mice exposed to ISO on PND 6 (Kodama et al., 2011). Differences between these previous findings and those from the current study include the species studied in Stratmann et al. (2009) and the fact that gender effects were not reported. The contextual fear conditioning deficits observed in Kodama et al. (2011) were in adolescent males, not adult females. However, when taken together, the research indicates neonatal ISO exposure may impair fear conditioning in rodents later in life.

In the current experiment, female ISO-mice also displayed initial hyperactivity during re-testing in the 1 hr locomotor activity test as adults. It is unlikely the social

approach and contextual fear conditioning differences were a result of this hyperactivity. No differences were observed in total distance traveled or chamber entries during social approach testing, which suggest the findings did not result from the females' activity levels. Hyperactivity within contextual fear conditioning could result in lower freezing behaviors regardless of normal contextual memory. This seems unlikely in the current study given that the female ISO-treated mice displayed comparable freezing behaviors to control mice during baseline measures and auditory-cue fear conditioning.

The lack of an influence of postnatal ISO exposure on MWM performance in the current study is notable, although consistent with previous findings in the mouse. Rats neonatally exposed to ISO exhibited impaired MWM performance as adolescents and adults, but not juveniles (Stratmann, Sall, et al., 2009). Separate studies also reported impaired MWM performance in adolescent and young adult rats neonatally exposed to ISO (Rothstein et al., 2008; Stratmann, May, et al., 2009). The current negative findings replicate those of previous mouse studies. No differences in MWM performance were observed in mice as juveniles or young adults following ISO exposure on PND 6 (Loepke et al., 2009). Behavioral disturbances in the mouse are likely induced by neonatal ISO exposure, given the differences in activity, social investigation, and conditioned fear, although gender by drug interactions may be important determinants of the types of impairments that are observed. However, the MWM does not appear to be sensitive for detecting developmental cognitive effects of ISO exposure in mice.

Based on the acute neurodegenerative effects of developmental ISO exposure observed in the current experiments, we would expect to see altered cell densities in adult mouse brains exposed to a similar ISO protocol. However, Loepke et al. (2009) reported

no difference in adult neuronal densities in 12-week-old mice following 1.5% ISO exposure for 6 hr on PND 7 despite an acute increase in cells positively stained with the neuroapoptosis marker AC3. This study also reported no differences in locomotor activity and spatial learning and memory performance between ISO-treated mice and controls. This suggests the plasticity of the infant mouse brain may be able to recover from the insult of a single, albeit long in duration, exposure to ISO on PND 7. Given the acute neuroapoptosis and behavioral impairments observed in the current study following multiple ISO exposures across 3 days, it is likely multiple exposures to ISO, which include exposures prior to PND 7, induce chronic changes to the developing mouse brain that would remain evident into adulthood.

The current study did not exhibit great evidence of behavioral dysfunction following neonatal ISO exposure while confirming ISO exposure alone deletes large numbers of neurons in the immature brain. Disruptions in activity levels and cognition were observed, respectively, in juvenile male and adult female mice exposed as neonates to ISO along with slight fine motor coordination and anxiety-related disruptions during adolescence. However, the majority of behavioral analyses revealed no differences between exposure groups. This suggests recovery of function possibly due to the plasticity of the developing mouse brain. Current clinical pediatric anesthesia protocols, however, combine GABAmimetics like ISO with other GABAmimetics and NMDA-antagonists such as midazolam and nitrous oxide (N_2O). The combined neuroapoptotic effect of GABA-potentiation and NMDA blockade is far greater than that of either action alone. While few cognitive disruptions were observed in the present study following multiple exposures to ISO alone, the functional impact of a single exposure to the triple

cocktail of ISO, midazolam, and N_2O in the rat is robust (Jevtovic-Todorovic et al., 2003). Multiple exposures to the triple anesthetic cocktail would likely produce substantial cognitive deficits in rodents. The current findings reinforce the need for further investigation of the impact of multiple anesthesia exposures given the clinical evidence of an association between the increased risk of LD and multiple exposures to anesthesia during the brain growth spurt period in children (Flick et al., 2011; Wilder et al., 2009).

CONCLUSION

These findings confirm that neonatal exposure to an anesthetic agent induces exaggerated cell death during development and is associated with long-term behavioral dysfunctions. Isoflurane heightened levels of neuroapoptosis, suggesting possible alteration of neural circuitry, and produced mild neuromotor, emotional, social and cognitive deficits, most often in a gender-dependent manner. The results from the present work stress the importance of further studies of this kind and provide the grounds for developing adjunctive therapies for preventing the neuroapoptosis and ensuing behavioral impairments induced by exposure to anesthetic agents during early development.

REFERENCES

Aggleton, J. P., O'Mara, S. M., Vann, S. D., Wright, N. F., Tsanov, M., & Erichsen, J. T. (2010). Hippocampal-anterior thalamic pathways for memory: uncovering a network of direct and indirect actions. *Eur J Neurosci, 31*(12), 2292-2307. doi: 10.1111/j.1460-9568.2010.07251.x

Altay, O., Hasegawa, Y., Sherchan, P., Suzuki, H., Khatibi, N. H., Tang, J., & Zhang, J. H. (2012). Isoflurane delays the development of early brain injury after subarachnoid hemorrhage through sphingosine-related pathway activation in mice. *Crit Care Med*. doi: 10.1097/CCM.0b013e3182474bc1

Ball, C., & Westhorpe, R. N. (2007). Isoflurane. *Anaesth Intensive Care, 35*(4), 467.

Bekker, A., Shah, R., Quartermain, D., Li, Y. S., & Blanck, T. (2006). Isoflurane preserves spatial working memory in adult mice after moderate hypoxia. *Anesth Analg, 102*(4), 1134-1138. doi: 10.1213/01.ane.0000198637.36539.c1

Bercker, S., Bert, B., Bittigau, P., Felderhoff-Muser, U., Buhrer, C., Ikonomidou, C., . . . Kerner, T. (2009). Neurodegeneration in newborn rats following propofol and sevoflurane anesthesia. *Neurotox Res, 16*(2), 140-147. doi: 10.1007/s12640-009-9063-8

Bianchi, S. L., Tran, T., Liu, C., Lin, S., Li, Y., Keller, J. M., . . . Eckenhoff, M. F. (2008). Brain and behavior changes in 12-month-old Tg2576 and nontransgenic mice exposed to anesthetics. *Neurobiol Aging, 29*(7), 1002-1010. doi: 10.1016/j.neurobiolaging.2007.02.009

Bittigau, P., Sifringer, M., Genz, K., Reith, E., Pospischil, D., Govindarajalu, S., . . . Ikonomidou, C. (2002). Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc Natl Acad Sci U S A, 99*(23), 15089-15094. doi: 10.1073/pnas.222550499

Briner, A., De Roo, M., Dayer, A., Muller, D., Habre, W., & Vutskits, L. (2010). Volatile anesthetics rapidly increase dendritic spine density in the rat medial prefrontal cortex during synaptogenesis. *Anesthesiology, 112*(3), 546-556. doi: 10.1097/ALN.0b013e3181cd7942

Bryan, K. K., Lee, H.-g., Perry, G., Smith, M. A., & Casadesus, G. (2009). Transgenic Mouse Models of Alzheimer's Disease: Behavioral Testing and Considerations. In J. J. Buccafusco (Ed.), *Methods of Behavior Analysis in Neuroscience* (2nd ed., pp. 1-18). Boca Raton, FL: CRC Press, Taylor & Francis Group, LLC.

Butterfield, N. N., Graf, P., Ries, C. R., & MacLeod, B. A. (2004). The Effect of Repeated Isoflurane Anesthesia on Spatial and Psychomotor Performance in Young and Aged Mice. *Anesthesia & Analgesia,* 1305-1311. doi: 10.1213/01.ane.0000108484.91089.13

Callaway, J. K., Jones, N. C., & Royse, C. F. (2012). Isoflurane induces cognitive deficits in the Morris water maze task in rats. *Eur J Anaesthesiol*. doi: 10.1097/EJA.0b013e32835103c1

Cattano, D., Young, C., Straiko, M. M., & Olney, J. W. (2008). Subanesthetic doses of propofol induce neuroapoptosis in the infant mouse brain. *Anesth Analg, 106*(6), 1712-1714. doi: 10.1213/ane.0b013e318172ba0a

Chen, H., Burris, M., Fajilan, A., Spagnoli, F., Tang, J., & Zhang, J. H. (2011). Prolonged Exposure to Isoflurane Ameliorates Infarction Severity in the Rat Pup Model of Neonatal Hypoxia-Ischemia. *Transl Stroke Res, 2*(3), 382-390. doi: 10.1007/s12975-011-0081-5

Clarren, S. K., Alvord, E. C., Jr., Sumi, S. M., Streissguth, A. P., & Smith, D. W. (1978). Brain malformations related to prenatal exposure to ethanol. *J Pediatr, 92*(1), 64-67.

Cohen, J., Dore, C., Robaire, B., & Ruf, K. B. (1984). Plasma concentrations of free 5 alpha-androstane-3 alpha, 17 beta-diol and related gonadal steroids during spontaneous and induced sexual maturation in the female rat. *Biol Reprod, 30*(1), 105-111.

Cote, C. J., Lerman, J., Ward, R. M., Lugo, R. A., & Goudsouzian, N. (2009). Pharmacokinetics and pharmacology of drugs used in children. In C. J. Cote, J. Lerman & I. D. Todres (Eds.), *A Practice of Anesthesia for Infants and Children* (Fourth ed., pp. 89-146). Philadelphia, PA: Saunders Elsevier.

Crosby, C., Culley, D. J., Baxter, M. G., Yukhananov, R., & Crosby, G. (2005). Spatial memory performance 2 weeks after general anesthesia in adult rats. *Anesth Analg, 101*(5), 1389-1392. doi: 10.1213/01.ANE.0000180835.72669.AD

Culley, D. J., Baxter, M. G., Crosby, C. A., Yukhananov, R., & Crosby, G. (2004). Impaired acquisition of spatial memory 2 weeks after isoflurane and isoflurane-nitrous oxide anesthesia in aged rats. *Anesth Analg, 99*(5), 1393-1397; table of contents. doi: 10.1213/01.ANE.0000135408.14319.CC

D'Mello, S. R., Kuan, C. Y., Flavell, R. A., & Rakic, P. (2000). Caspase-3 is required for apoptosis-associated DNA fragmentation but not for cell death in neurons deprived of potassium. *J Neurosci Res, 59*(1), 24-31.

Dallasen, R. M., Bowman, J. D., & Xu, Y. (2011). Isoflurane Does Not Cause Neuroapoptosis but Reduces Astroglial Processes in Young Adult Mice. *Med Gas Res, 1*(1), 27. doi: 10.1186/2045-9912-1-27

DeFrances, C. J., Cullen, K. A., & Kozak, L. J. (2007). National Hospital Discharge Survey: 2005 annual summary with detailed diagnosis and procedure data. *Vital Health Stat 13*(165), 1-209.

Diaz Rodriguez, E., Diaz Lopez, B., Debeljuk, L., Esquifino Parras, A. I., Arce Fraguas, A., & Marin Fernandez, B. (1999). Developmental changes of hypothalamic, pituitary and striatal tachykinins in response to testosterone: influence of prenatal melatonin. *Peptides, 20*(4), 501-508.

Dickinson, R., de Sousa, S. L., Lieb, W. R., & Franks, N. P. (2002). Selective synaptic actions of thiopental and its enantiomers. *Anesthesiology, 96*(4), 884-892.

Dickinson, R., Peterson, B. K., Banks, P., Simillis, C., Martin, J. C., Valenzuela, C. A., . . . Franks, N. P. (2007). Competitive inhibition at the glycine site of the N-methyl-D-aspartate receptor by the anesthetics xenon and isoflurane: evidence from molecular modeling and electrophysiology. *Anesthesiology, 107*(5), 756-767. doi: 10.1097/01.anes.0000287061.77674.71

Dikranian, K., Ishimaru, M. J., Tenkova, T., Labruyere, J., Qin, Y. Q., Ikonomidou, C., & Olney, J. W. (2001). Apoptosis in the in vivo mammalian forebrain. *Neurobiol Dis, 8*(3), 359-379. doi: 10.1006/nbdi.2001.0411

Dikranian, K., Qin, Y. Q., Labruyere, J., Nemmers, B., & Olney, J. W. (2005). Ethanol-induced neuroapoptosis in the developing rodent cerebellum and related brain stem structures. *Brain Res Dev Brain Res, 155*(1), 1-13. doi: 10.1016/j.devbrainres.2004.11.005

DiMaggio, C., Sun, L. S., Kakavouli, A., Byrne, M. W., & Li, G. (2009). A retrospective cohort study of the association of anesthesia and hernia repair surgery with behavioral and developmental disorders in young children. *J Neurosurg Anesthesiol, 21*(4), 286-291. doi: 10.1097/ANA.0b013e3181a71f11

Dobbing, J., & Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Hum Dev, 3*(1), 79-83.

Eger, E. I., 2nd. (1981). Isoflurane: A review. *Anesthesiology, 55*, 559-576.

Elsersy, H., Sheng, H., Lynch, J. R., Moldovan, M., Pearlstein, R. D., & Warner, D. S. (2004). Effects of isoflurane versus fentanyl-nitrous oxide anesthesia on long-term outcome from severe forebrain ischemia in the rat. *Anesthesiology, 100*(5), 1160-1166.

File, S. E. (1986a). Behavioral changes persisting into adulthood after neonatal benzodiazepine administration in the rat. *Neurobehav Toxicol Teratol, 8*(5), 453-461.

File, S. E. (1986b). The effects of neonatal administration of clonazepam on passive avoidance and on social, aggressive and exploratory behavior of adolescent male rats. *Neurobehav Toxicol Teratol, 8*(5), 447-452.

File, S. E. (1986c). Effects of neonatal administration of diazepam and lorazepam on performance of adolescent rats in tests of anxiety, aggression, learning and convulsions. *Neurobehav Toxicol Teratol, 8*(3), 301-306.

File, S. E. (1987). Diazepam and caffeine administration during the first week of life: changes in neonatal and adolescent behavior. *Neurotoxicol Teratol, 9*(1), 9-16.

Flick, R. P., Katusic, S. K., Colligan, R. C., Wilder, R. T., Voigt, R. G., Olson, M. D., . . . Warner, D. O. (2011). Cognitive and behavioral outcomes after early exposure to anesthesia and surgery. *Pediatrics, 128*(5), e1053-1061. doi: 10.1542/peds.2011-0351

Forman, S. A., & Chin, V. A. (2008). General anesthetics and molecular mechanisms of unconsciousness. *Int Anesthesiol Clin, 46*(3), 43-53. doi: 10.1097/AIA.0b013e3181755da5

Franks, N. P., & Lieb, W. R. (1994). Molecular and cellular mechanisms of general anaesthesia. *Nature, 367*(6464), 607-614. doi: 10.1038/367607a0

Fredriksson, A., & Archer, T. (2003). Hyperactivity following postnatal NMDA antagonist treatment: reversal by D-amphetamine. *Neurotox Res, 5*(7), 549-564.

Fredriksson, A., & Archer, T. (2004). Neurobehavioural deficits associated with apoptotic neurodegeneration and vulnerability for ADHD. *Neurotox Res, 6*(6), 435-456.

Fredriksson, A., Archer, T., Alm, H., Gordh, T., & Eriksson, P. (2004). Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration. *Behav Brain Res, 153*(2), 367-376. doi: 10.1016/j.bbr.2003.12.026

Fredriksson, A., Ponten, E., Gordh, T., & Eriksson, P. (2007). Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. *Anesthesiology, 107*(3), 427-436. doi: 10.1097/01.anes.0000278892.62305.9c

Harris, R. A., Proctor, W. R., McQuilkin, S. J., Klein, R. L., Mascia, M. P., Whatley, V., . . . Dunwiddie, T. V. (1995). Ethanol increases GABAA responses in cells stably transfected with receptor subunits. *Alcohol Clin Exp Res, 19*(1), 226-232.

Hemmings, H. C., Jr. (2009). Sodium channels and the synaptic mechanisms of inhaled anaesthetics. *Br J Anaesth, 103*(1), 61-69. doi: 10.1093/bja/aep144

Hemmings, H. C., Jr., Yan, W., Westphalen, R. I., & Ryan, T. A. (2005). The general anesthetic isoflurane depresses synaptic vesicle exocytosis. *Mol Pharmacol, 67*(5), 1591-1599. doi: 10.1124/mol.104.003210

Herring, B. E., Xie, Z., Marks, J., & Fox, A. P. (2009). Isoflurane inhibits the neurotransmitter release machinery. *J Neurophysiol, 102*(2), 1265-1273. doi: 10.1152/jn.00252.2009

Hertle, D., Beynon, C., Zweckberger, K., Vienenkotter, B., Jung, C. S., Kiening, K., . . . Sakowitz, O. W. (2012). Influence of isoflurane on neuronal death and outcome in a rat model of traumatic brain injury. *Acta Neurochir Suppl, 114*, 383-386. doi: 10.1007/978-3-7091-0956-4_74

Hoffman, P. L., Rabe, C. S., Moses, F., & Tabakoff, B. (1989). N-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J Neurochem, 52*(6), 1937-1940.

Holmes, L. B., Harvey, E. A., Coull, B. A., Huntington, K. B., Khoshbin, S., Hayes, A. M., & Ryan, L. M. (2001). The teratogenicity of anticonvulsant drugs. *N Engl J Med, 344*(15), 1132-1138. doi: 10.1056/NEJM200104123441504

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, D. F., Koch, C., Genz, K., . . . Olney, J. W. (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science, 287*(5455), 1056-1060.

Ikonomidou, C., Bittigau, P., Koch, C., Genz, K., Hoerster, F., Felderhoff-Mueser, U., . . . Olney, J. W. (2001). Neurotransmitters and apoptosis in the developing brain. *Biochem Pharmacol, 62*(4), 401-405.

Ikonomidou, C., Bosch, F. H., Miksa, M., Bittigau, P., Vockler, J., Dikranian, K., . . . Olney, J. W. (1999). Blockade of NMDA Receptors and Apoptotic Neurodegeneration in the Developing Brain. *Science, 283*(5398), 70-74. doi: 10.1126/science.283.5398.70

Ikonomidou, C., Stefovska, V., & Turski, L. (2000). Neuronal death enhanced by N-methyl-D-aspartate antagonists. *Proc Natl Acad Sci U S A, 97*(23), 12885-12890. doi: 10.1073/pnas.220412197

Irifune, M., Takarada, T., Shimizu, Y., Endo, C., Katayama, S., Dohi, T., & Kawahara, M. (2003). Propofol-induced anesthesia in mice is mediated by gamma-aminobutyric acid-A and excitatory amino acid receptors. *Anesth Analg, 97*(2), 424-429, table of contents.

Ishimaru, M. J., Ikonomidou, C., Tenkova, T. I., Der, T. C., Dikranian, K., Sesma, M. A., & Olney, J. W. (1999). Distinguishing excitotoxic from apoptotic neurodegeneration in the developing rat brain. *J Comp Neurol, 408*(4), 461-476.

Istaphanous, G. K., Howard, J., Nan, X., Hughes, E. A., McCann, J. C., McAuliffe, J. J., . . . Loepke, A. W. (2011). Comparison of the neuroapoptotic properties of equipotent anesthetic concentrations of desflurane, isoflurane, or sevoflurane in

neonatal mice. *Anesthesiology, 114*(3), 578-587. doi: 10.1097/ALN.0b013e3182084a70

Jevtovic-Todorovic, V., Hartman, R. E., Izumi, Y., Benshoff, N. D., Dikranian, K., Zorumski, C. F., . . . Wozniak, D. F. (2003). Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci, 23*(3), 876-882.

Jia, F., Yue, M., Chandra, D., Homanics, G. E., Goldstein, P. A., & Harrison, N. L. (2008). Isoflurane is a potent modulator of extrasynaptic GABA(A) receptors in the thalamus. *J Pharmacol Exp Ther, 324*(3), 1127-1135. doi: 10.1124/jpet.107.134569

Jiang, H., Huang, Y., Xu, H., Sun, Y., Han, N., & Li, Q. F. (2012). Hypoxia inducible factor-1alpha is involved in the neurodegeneration induced by isoflurane in the brain of neonatal rats. *J Neurochem, 120*(3), 453-460. doi: 10.1111/j.1471-4159.2011.07589.x

Johnson, S. A., Young, C., & Olney, J. W. (2008). Isoflurane-induced neuroapoptosis in the developing brain of nonhypoglycemic mice. *J Neurosurg Anesthesiol, 20*(1), 21-28. doi: 10.1097/ANA.0b013e3181271850

Jones, K. L., Lacro, R. V., Johnson, K. A., & Adams, J. (1989). Pattern of malformations in the children of women treated with carbamazepine during pregnancy. *N Engl J Med, 320*(25), 1661-1666. doi: 10.1056/NEJM198906223202505

Jones, K. L., & Smith, D. W. (1975). The fetal alcohol syndrome. *Teratology, 12*(1), 1-10. doi: 10.1002/tera.1420120102

Julien, R. M. (2005). *A Primer of Drug Action: A Comprehensive Guide to the Actions, Uses, and Side Effects of Psychoactive Drugs* (Tenth ed.). New York, NY: Worth Publishers.

Kalkman, C. J., Peelen, L., Moons, K. G., Veenhuizen, M., Bruens, M., Sinnema, G., & de Jong, T. P. (2009). Behavior and development in children and age at the time of first anesthetic exposure. *Anesthesiology, 110*(4), 805-812. doi: 10.1097/ALN.0b013e31819c7124

Kawabe, K., Iwasaki, T., & Ichitani, Y. (2007). Repeated treatment with N-methyl-d-aspartate antagonists in neonatal, but not adult, rats causes long-term deficits of radial-arm maze learning. *Brain Res, 1169*, 77-86. doi: 10.1016/j.brainres.2007.06.062

Kawaguchi, M., Drummond, J. C., Cole, D. J., Kelly, P. J., Spurlock, M. P., & Patel, P. M. (2004). Effect of isoflurane on neuronal apoptosis in rats subjected to focal cerebral ischemia. *Anesth Analg, 98*(3), 798-805, table of contents.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer, 26*(4), 239-257.

Khatibi, N. H., Ma, Q., Rolland, W., Ostrowski, R., Fathali, N., Martin, R., . . . Zhang, J. H. (2011). Isoflurane posttreatment reduces brain injury after an intracerebral hemorrhagic stroke in mice. *Anesth Analg, 113*(2), 343-348. doi: 10.1213/ANE.0b013e31821f9524

Khuchua, Z., Wozniak, D. F., Bardgett, M. E., Yue, Z., McDonald, M., Boero, J., . . . Strauss, A. W. (2003). Deletion of the N-terminus of murine map2 by gene targeting disrupts hippocampal ca1 neuron architecture and alters contextual memory. *Neuroscience, 119*(1), 101-111.

Kodama, M., Satoh, Y., Otsubo, Y., Araki, Y., Yonamine, R., Masui, K., & Kazama, T. (2011). Neonatal desflurane exposure induces more robust neuroapoptosis than do isoflurane and sevoflurane and impairs working memory. *Anesthesiology, 115*(5), 979-991. doi: 10.1097/ALN.0b013e318234228b

Kolb, B., & Gibb, R. (2007). Brain plasticity and recovery from early cortical injury. *Dev Psychobiol, 49*(2), 107-118. doi: 10.1002/dev.20199

Kopp Lugli, A., Yost, C. S., & Kindler, C. H. (2009). Anaesthetic mechanisms: update on the challenge of unravelling the mystery of anaesthesia. *Eur J Anaesthesiol, 26*(10), 807-820. doi: 10.1097/EJA.0b013e32832d6b0f

Lazar, N. L., Neufeld, R. W., & Cain, D. P. (2011). Contribution of nonprimate animal models in understanding the etiology of schizophrenia. *J Psychiatry Neurosci, 36*(4), E5-29. doi: 10.1503/jpn.100054

Lee, J. J., Li, L., Jung, H. H., & Zuo, Z. (2008). Postconditioning with isoflurane reduced ischemia-induced brain injury in rats. *Anesthesiology, 108*(6), 1055-1062. doi: 10.1097/ALN.0b013e3181730257

Li, L., & Zuo, Z. (2009). Isoflurane preconditioning improves short-term and long-term neurological outcome after focal brain ischemia in adult rats. *Neuroscience, 164*(2), 497-506. doi: 10.1016/j.neuroscience.2009.08.011

Li, Y., Liang, G., Wang, S., Meng, Q., Wang, Q., & Wei, H. (2007). Effects of fetal exposure to isoflurane on postnatal memory and learning in rats. *Neuropharmacology, 53*(8), 942-950. doi: 10.1016/j.neuropharm.2007.09.005

Liang, G., Ward, C., Peng, J., Zhao, Y., Huang, B., & Wei, H. (2010). Isoflurane causes greater neurodegeneration than an equivalent exposure of sevoflurane in the developing brain of neonatal mice. *Anesthesiology, 112*(6), 1325-1334. doi: 10.1097/ALN.0b013e3181d94da5

Lin, D., & Zuo, Z. (2011). Isoflurane induces hippocampal cell injury and cognitive impairments in adult rats. *Neuropharmacology, 61*(8), 1354-1359. doi: 10.1016/j.neuropharm.2011.08.011

Lingamaneni, R., Birch, M. L., & Hemmings, H. C., Jr. (2001). Widespread inhibition of sodium channel-dependent glutamate release from isolated nerve terminals by isoflurane and propofol. *Anesthesiology, 95*(6), 1460-1466.

Liu, W., Xu, J., Wang, H., Xu, C., Ji, C., Wang, Y., . . . Yue, Y. (2012). Isoflurane-induced spatial memory impairment by a mechanism independent of amyloid-beta levels and tau protein phosphorylation changes in aged rats. *Neurol Res, 34*(1), 3-10. doi: 10.1179/1743132811y.0000000047

Loepke, A. W., Istaphanous, G. K., McAuliffe, J. J., 3rd, Miles, L., Hughes, E. A., McCann, J. C., . . . Danzer, S. C. (2009). The effects of neonatal isoflurane exposure in mice on brain cell viability, adult behavior, learning, and memory. *Anesth Analg, 108*(1), 90-104. doi: 10.1213/ane.0b013e31818cdb29

Loepke, A. W., McCann, J. C., Kurth, C. D., & McAuliffe, J. J. (2006). The physiologic effects of isoflurane anesthesia in neonatal mice. *Anesth Analg, 102*(1), 75-80. doi: 10.1213/01.ANE.0000181102.92729.B8

Lonnquist, P.-A. (2002). Management of the neonate: Anesthetic considerations and postoperative management. In B. Bissonnette & B. J. Dalens (Eds.), *Pediatric Anesthesia: Principles and Practice* (pp. 995-1030). United States of America: McGraw-Hill.

Lovinger, D. M., White, G., & Weight, F. F. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science, 243*(4899), 1721-1724.

McAuliffe, J. J., Joseph, B., & Vorhees, C. V. (2007). Isoflurane-delayed preconditioning reduces immediate mortality and improves striatal function in adult mice after neonatal hypoxia-ischemia. *Anesth Analg, 104*(5), 1066-1077, tables of contents. doi: 10.1213/01.ane.0000260321.62377.74

McAuliffe, J. J., Loepke, A. W., Miles, L., Joseph, B., Hughes, E., & Vorhees, C. V. (2009). Desflurane, isoflurane, and sevoflurane provide limited neuroprotection against neonatal hypoxia-ischemia in a delayed preconditioning paradigm. *Anesthesiology, 111*(3), 533-546. doi: 10.1097/ALN.0b013e3181b060d3

Miczek, K. A., Maxson, S. C., Fish, E. W., & Faccidomo, S. (2001). Aggressive behavioral phenotypes in mice. *Behav Brain Res, 125*(1-2), 167-181.

Ming, Z., Knapp, D. J., Mueller, R. A., Breese, G. R., & Criswell, H. E. (2001). Differential modulation of GABA- and NMDA-gated currents by ethanol and isoflurane in cultured rat cerebral cortical neurons. *Brain Res, 920*(1-2), 117-124.

Moller, J. T., Cluitmans, P., Rasmussen, L. S., Houx, P., Rasmussen, H., Canet, J., . . . Gravenstein, J. S. (1998). Long-term postoperative cognitive dysfunction in the elderly ISPOCD1 study. ISPOCD investigators. International Study of Post-Operative Cognitive Dysfunction. *Lancet, 351*(9106), 857-861.

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., . . . Crawley, J. N. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav, 3*(5), 287-302. doi: 10.1111/j.1601-1848.2004.00076.x

Nadler, J. J., Moy, S. S., Dold, G., Trang, D., Simmons, N., Perez, A., . . . Crawley, J. N. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes Brain Behav, 3*(5), 303-314. doi: 10.1111/j.1601-183X.2004.00071.x

Naert, A., Callaerts-Vegh, Z., & D'Hooge, R. (2011). Nocturnal hyperactivity, increased social novelty preference and delayed extinction of fear responses in post-weaning socially isolated mice. *Brain Res Bull, 85*(6), 354-362. doi: 10.1016/j.brainresbull.2011.03.027

Nishikawa, K., & Harrison, N. L. (2003). The actions of sevoflurane and desflurane on the gamma-aminobutyric acid receptor type A: effects of TM2 mutations in the alpha and beta subunits. *Anesthesiology, 99*(3), 678-684.

Olney, J. (1974). Toxxic effects of glutamate and related amino acids on the developing central nervous system. In W. L. Nyhan (Ed.), *Heritable Disorders of Amino Acid Metabolism* (pp. 501-509). New York, NY: Wiley.

Olney, J. W., Tenkova, T., Dikranian, K., Muglia, L. J., Jermakowicz, W. J., D'Sa, C., & Roth, K. A. (2002). Ethanol-induced caspase-3 activation in the in vivo developing mouse brain. *Neurobiol Dis, 9*(2), 205-219. doi: 10.1006/nbdi.2001.0475

Olney, J. W., Tenkova, T., Dikranian, K., Qin, Y. Q., Labruyere, J., & Ikonomidou, C. (2002). Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Brain Res Dev Brain Res, 133*(2), 115-126.

Ouyang, W., Herold, K. F., & Hemmings, H. C., Jr. (2009). Comparative effects of halogenated inhaled anesthetics on voltage-gated Na+ channel function. *Anesthesiology, 110*(3), 582-590. doi: 10.1097/ALN.0b013e318197941e

Palanisamy, A., Baxter, M. G., Keel, P. K., Xie, Z., Crosby, G., & Culley, D. J. (2011). Rats exposed to isoflurane in utero during early gestation are behaviorally abnormal as adults. *Anesthesiology, 114*(3), 521-528. doi: 10.1097/ALN.0b013e318209aa71

Perucho, J., Casarejos, M. J., Gomez, A., Solano, R. M., de Yebenes, G., & Mena, M. A. (2012). Trehalose protects from aggravation of amyloid pathology induced by isoflurane anesthesia in APPswe mutant mice. *Curr Alzheimer Res*.

Perucho, J., Rubio, I., Casarejos, M. J., Gomez, A., Rodriguez-Navarro, J. A., Solano, R. M., . . . Mena, M. A. (2010). Anesthesia with isoflurane increases amyloid pathology in mice models of Alzheimer's disease. *J Alzheimers Dis, 19*(4), 1245-1257. doi: 10.3233/JAD-2010-1318

Planel, E., Bretteville, A., Liu, L., Virag, L., Du, A. L., Yu, W. H., . . . Duff, K. E. (2009). Acceleration and persistence of neurofibrillary pathology in a mouse model of tauopathy following anesthesia. *FASEB J, 23*(8), 2595-2604. doi: 10.1096/fj.08-122424

Rammes, G., Starker, L. K., Haseneder, R., Berkmann, J., Plack, A., Zieglgansberger, W., . . . Blobner, M. (2009). Isoflurane anaesthesia reversibly improves cognitive function and long-term potentiation (LTP) via an up-regulation in NMDA receptor 2B subunit expression. *Neuropharmacology, 56*(3), 626-636. doi: 10.1016/j.neuropharm.2008.11.002

Rau, V., Iyer, S. V., Oh, I., Chandra, D., Harrison, N., Eger, E. I., 2nd, . . . Sonner, J. M. (2009). Gamma-aminobutyric acid type A receptor alpha 4 subunit knockout mice are resistant to the amnestic effect of isoflurane. *Anesth Analg, 109*(6), 1816-1822. doi: 10.1213/ANE.0b013e3181bf6ae6

Robb, G. W., Amann, R. P., & Killian, G. J. (1978). Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J Reprod Fertil, 54*(1), 103-107.

Rothstein, S., Simkins, T., & Nunez, J. L. (2008). Response to neonatal anesthesia: effect of sex on anatomical and behavioral outcome. *Neuroscience, 152*(4), 959-969. doi: 10.1016/j.neuroscience.2008.01.027

Roxo, M. R., Franceschini, P. R., Zubaran, C., Kleber, F. D., & Sander, J. W. (2011). The limbic system conception and its historical evolution. *ScientificWorldJournal, 11*, 2428-2441. doi: 10.1100/2011/157150

Sasaoka, N., Kawaguchi, M., Kawaraguchi, Y., Nakamura, M., Konishi, N., Patel, H., . . . Furuya, H. (2009). Isoflurane exerts a short-term but not a long-term preconditioning effect in neonatal rats exposed to a hypoxic-ischaemic neuronal injury. *Acta Anaesthesiol Scand, 53*(1), 46-54. doi: 10.1111/j.1399-6576.2008.01822.x

Satomoto, M., Satoh, Y., Terui, K., Miyao, H., Takishima, K., Ito, M., & Imaki, J. (2009). Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. *Anesthesiology, 110*(3), 628-637. doi: 10.1097/ALN.0b013e3181974fa2

Scallet, A. C., Schmued, L. C., Slikker, W., Jr., Grunberg, N., Faustino, P. J., Davis, H., . . . Hanig, J. P. (2004). Developmental neurotoxicity of ketamine: morphometric confirmation, exposure parameters, and multiple fluorescent labeling of apoptotic neurons. *Toxicol Sci, 81*(2), 364-370. doi: 10.1093/toxsci/kfh224

Schmued, L. C., Albertson, C., & Slikker, W., Jr. (1997). Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res, 751*(1), 37-46.

Schroeder, H., Humbert, A. C., Desor, D., & Nehlig, A. (1997). Long-term consequences of neonatal exposure to diazepam on cerebral glucose utilization, learning, memory and anxiety. *Brain Res, 766*(1-2), 142-152.

Sharma, S., Rakoczy, S., & Brown-Borg, H. (2010). Assessment of spatial memory in mice. *Life Sci, 87*(17-18), 521-536. doi: 10.1016/j.lfs.2010.09.004

Shiraishi, M., & Harris, R. A. (2004). Effects of alcohols and anesthetics on recombinant voltage-gated Na+ channels. *J Pharmacol Exp Ther, 309*(3), 987-994. doi: 10.1124/jpet.103.064063

Silverman, J. L., Yang, M., Lord, C., & Crawley, J. N. (2010). Behavioural phenotyping assays for mouse models of autism. *Nat Rev Neurosci, 11*(7), 490-502. doi: 10.1038/nrn2851

Slikker, W., Jr., Zou, X., Hotchkiss, C. E., Divine, R. L., Sadovova, N., Twaddle, N. C., . . . Wang, C. (2007). Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol Sci, 98*(1), 145-158. doi: 10.1093/toxsci/kfm084

Speidel, B. D., & Meadow, S. R. (1972). Maternal epilepsy and abnormalities of the fetus and newborn. *Lancet, 2*(7782), 839-843.

Stabernack, C., Sonner, J. M., Laster, M., Zhang, Y., Xing, Y., Sharma, M., & Eger, E. I., 2nd. (2003). Spinal N-methyl-d-aspartate receptors may contribute to the immobilizing action of isoflurane. *Anesth Analg, 96*(1), 102-107, table of contents.

Statler, K. D., Alexander, H., Vagni, V., Holubkov, R., Dixon, C. E., Clark, R. S., . . . Kochanek, P. M. (2006). Isoflurane exerts neuroprotective actions at or near the time of severe traumatic brain injury. *Brain Res, 1076*(1), 216-224. doi: 10.1016/j.brainres.2005.12.106

Stefovska, V. G., Uckermann, O., Czuczwar, M., Smitka, M., Czuczwar, P., Kis, J., . . . Ikonomidou, C. (2008). Sedative and anticonvulsant drugs suppress postnatal neurogenesis. *Ann Neurol, 64*(4), 434-445. doi: 10.1002/ana.21463

Straiko, M. M., Young, C., Cattano, D., Creeley, C. E., Wang, H., Smith, D. J., . . . Olney, J. W. (2009). Lithium protects against anesthesia-induced developmental

neuroapoptosis. *Anesthesiology, 110*(4), 862-868. doi: 10.1097/ALN.0b013e31819b5eab

Stratmann, G., May, L. D., Sall, J. W., Alvi, R. S., Bell, J. S., Ormerod, B. K., . . . Firouzian, A. (2009). Effect of hypercarbia and isoflurane on brain cell death and neurocognitive dysfunction in 7-day-old rats. *Anesthesiology, 110*(4), 849-861. doi: 10.1097/ALN.0b013e31819c7140

Stratmann, G., Sall, J. W., Bell, J. S., Alvi, R. S., May, L. V., Ku, B., . . . Chiu, C. (2010). Isoflurane does not affect brain cell death, hippocampal neurogenesis, or long-term neurocognitive outcome in aged rats. *Anesthesiology, 112*(2), 305-315. doi: 10.1097/ALN.0b013e3181ca33a1

Stratmann, G., Sall, J. W., May, L. D., Bell, J. S., Magnusson, K. R., Rau, V., . . . Dai, R. (2009). Isoflurane differentially affects neurogenesis and long-term neurocognitive function in 60-day-old and 7-day-old rats. *Anesthesiology, 110*(4), 834-848. doi: 10.1097/ALN.0b013e31819c463d

Su, D., Zhao, Y., Wang, B., Li, W., Xiao, J., Chen, J., & Wang, X. (2011). Repeated but not single isoflurane exposure improved the spatial memory of young adult mice. *Acta Anaesthesiol Scand, 55*(4), 468-473. doi: 10.1111/j.1399-6576.2010.02385.x

Su, D., Zhao, Y., Wang, B., Xu, H., Li, W., Chen, J., & Wang, X. (2011). Isoflurane-Induced Spatial Memory Impairment in Mice is Prevented by the Acetylcholinesterase Inhibitor Donepezil. *PLoS One, 6*(11), e27632. doi: 10.1371/journal.pone.0027632

Tekin, S., & Cummings, J. L. (2002). Frontal-subcortical neuronal circuits and clinical neuropsychiatry: an update. *J Psychosom Res, 53*(2), 647-654.

Tomlin, S. L., Jenkins, A., Lieb, W. R., & Franks, N. P. (1998). Stereoselective effects of etomidate optical isomers on gamma-aminobutyric acid type A receptors and animals. *Anesthesiology, 88*(3), 708-717.

Valentim, A. M., Di Giminiani, P., Ribeiro, P. O., Rodrigues, P., Olsson, I. A., & Antunes, L. M. (2010). Lower isoflurane concentration affects spatial learning and neurodegeneration in adult mice compared with higher concentrations. *Anesthesiology, 113*(5), 1099-1108. doi: 10.1097/ALN.0b013e3181f79c7c

Van Dort, C. J., Baghdoyan, H. A., & Lydic, R. (2008). Neurochemical modulators of sleep and anesthetic states. *Int Anesthesiol Clin, 46*(3), 75-104. doi: 10.1097/AIA.0b013e318181a8ca

Vanini, G., Watson, C. J., Lydic, R., & Baghdoyan, H. A. (2008). Gamma-aminobutyric acid-mediated neurotransmission in the pontine reticular formation modulates hypnosis, immobility, and breathing during isoflurane anesthesia. *Anesthesiology, 109*(6), 978-988. doi: 10.1097/ALN.0b013e31818e3b1b

Wang, C., Sadovova, N., Hotchkiss, C., Fu, X., Scallet, A. C., Patterson, T. A., . . . Slikker, W., Jr. (2006). Blockade of N-methyl-D-aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture. *Toxicol Sci, 91*(1), 192-201. doi: 10.1093/toxsci/kfj144

Wang, S., Peretich, K., Zhao, Y., Liang, G., Meng, Q., & Wei, H. (2009). Anesthesia-induced neurodegeneration in fetal rat brains. *Pediatr Res, 66*(4), 435-440. doi: 10.1203/PDR.0b013e3181b3381b

Wei, H., Liang, G., & Yang, H. (2007). Isoflurane preconditioning inhibited isoflurane-induced neurotoxicity. *Neuroscience Letters, 425*(1), 59-62. doi: 10.1016/j.neulet.2007.08.011

West, J. R. (1987). Fetal alcohol-induced brain damage and the problem of determining temporal vulnerability: a review. *Alcohol Drug Res, 7*(5-6), 423-441.

Wilder, R. T., Flick, R. P., Sprung, J., Katusic, S. K., Barbaresi, W. J., Mickelson, C., . . . Warner, D. O. (2009). Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology, 110*(4), 796-804. doi: 10.1097/01.anes.0000344728.34332.5d

Wozniak, D. F., Hartman, R. E., Boyle, M. P., Vogt, S. K., Brooks, A. R., Tenkova, T., . . . Muglia, L. J. (2004). Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. *Neurobiol Dis, 17*(3), 403-414. doi: 10.1016/j.nbd.2004.08.006

Wozniak, D. F., Xiao, M., Xu, L., Yamada, K. A., & Ornitz, D. M. (2007). Impaired spatial learning and defective theta burst induced LTP in mice lacking fibroblast growth factor 14. *Neurobiol Dis, 26*(1), 14-26. doi: 10.1016/j.nbd.2006.11.014

Wu, X. S., Sun, J. Y., Evers, A. S., Crowder, M., & Wu, L. G. (2004). Isoflurane inhibits transmitter release and the presynaptic action potential. *Anesthesiology, 100*(3), 663-670.

Xie, Z., Culley, D. J., Dong, Y., Zhang, G., Zhang, B., Moir, R. D., . . . Tanzi, R. E. (2008). The common inhalation anesthetic isoflurane induces caspase activation and increases amyloid beta-protein level in vivo. *Ann Neurol, 64*(6), 618-627. doi: 10.1002/ana.21548

Xiong, L., Zheng, Y., Wu, M., Hou, L., Zhu, Z., Zhang, X., & Lu, Z. (2003). Preconditioning with isoflurane produces dose-dependent neuroprotection via activation of adenosine triphosphate-regulated potassium channels after focal cerebral ischemia in rats. *Anesth Analg, 96*(1), 233-237, table of contents.

Xu, Z., Dong, Y., Wu, X., Zhang, J., McAuliffe, S., Pan, C., . . . Xie, Z. (2011). The potential dual effects of anesthetic isoflurane on Abeta-induced apoptosis. *Curr Alzheimer Res, 8*(7), 741-752.

Yanai, J., Fares, F., Gavish, M., Greenfeld, Z., Katz, Y., Marcovici, G., . . . Weizman, A. (1989). Neural and behavioral alterations after early exposure to phenobarbital. *Neurotoxicology, 10*(3), 543-554.

Yon, J. H., Daniel-Johnson, J., Carter, L. B., & Jevtovic-Todorovic, V. (2005). Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. *Neuroscience, 135*(3), 815-827. doi: 10.1016/j.neuroscience.2005.03.064

Young, C., Jevtovic-Todorovic, V., Qin, Y. Q., Tenkova, T., Wang, H., Labruyere, J., & Olney, J. W. (2005). Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain. *Br J Pharmacol, 146*(2), 189-197. doi: 10.1038/sj.bjp.0706301

Young, C., & Olney, J. W. (2006). Neuroapoptosis in the infant mouse brain triggered by a transient small increase in blood alcohol concentration. *Neurobiol Dis, 22*(3), 548-554. doi: 10.1016/j.nbd.2005.12.015

Yuede, C. M., Wozniak, D. F., Creeley, C. E., Taylor, G. T., Olney, J. W., & Farber, N. B. (2010). Behavioral consequences of NMDA antagonist-induced neuroapoptosis in the infant mouse brain. *PLoS One, 5*(6), e11374. doi: 10.1371/journal.pone.0011374

Zhao, P., Peng, L., Li, L., Xu, X., & Zuo, Z. (2007). Isoflurane preconditioning improves long-term neurologic outcome after hypoxic-ischemic brain injury in neonatal rats. *Anesthesiology, 107*(6), 963-970. doi: 10.1097/01.anes.0000291447.21046.4d

Zhao, P., & Zuo, Z. (2004). Isoflurane preconditioning induces neuroprotection that is inducible nitric oxide synthase-dependent in neonatal rats. *Anesthesiology, 101*(3), 695-703.

Zhu, C., Gao, J., Karlsson, N., Li, Q., Zhang, Y., Huang, Z., . . . Blomgren, K. (2010). Isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells, and reduced neurogenesis in young, but not adult, rodents. *J Cereb Blood Flow Metab, 30*(5), 1017-1030. doi: 10.1038/jcbfm.2009.274

Zou, X., Patterson, T. A., Sadovova, N., Twaddle, N. C., Doerge, D. R., Zhang, X., . . . Wang, C. (2009). Potential neurotoxicity of ketamine in the developing rat brain. *Toxicol Sci, 108*(1), 149-158. doi: 10.1093/toxsci/kfn270

Zou, X., Sadovova, N., Patterson, T. A., Divine, R. L., Hotchkiss, C. E., Ali, S. F., . . . Wang, C. (2008). The effects of L-carnitine on the combination of, inhalation anesthetic-induced developmental, neuronal apoptosis in the rat frontal cortex. *Neuroscience*, *151*(4), 1053-1065. doi: 10.1016/j.neuroscience.2007.12.013