A Mechanistic Investigation of Anesthesia-Induced Spatial Learning Deficits in Aged Rats

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A MECHANISTIC INVESTIGATION OF ANESTHESIA-INDUCED SPATIAL LEARNING DEFICITS IN AGED RATS

By

Lana Jones Mawhinney

A DISSERTATION

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Coral Gables, Florida

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A MECHANISTIC INVESTIGATION OF ANESTHESIA-INDUCED SPATIAL LEARNING DEFICITS IN AGED RATS

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Anesthesia-induced spatial learning impairments in aged rats model postoperative cognitive dysfunction (POCD) in the elderly surgical population. Mechanisms underlying both normal age-related cognitive decline and anesthesia-induced spatial learning deficits in aged rats were investigated. With respect to the involvement of inflammasome activation and age-related cognitive decline, I hypothesized that the aged hippocampus exhibits an elevated activation of inflammasome components contributing to elevated levels of IL-1β in the aged brain. Age-related cognitive decline was identified in a subpopulation of male Fischer 344 rats. Activation of the NLRP1 inflammasome was elevated in the aged brain, contributing to spatial learning deficits in aged rats. With respect to anesthesia-induced spatial learning impairment in aged rats, I hypothesized that an increase in NR2B subunit in the hippocampus and cortex during and following isoflurane anesthesia exposure resulting in spatial learning impairment in aged rats via disruption of downstream signaling molecule, extracellular-signal regulated protein kinase (ERK). Anesthesia exposure resulted in chronic spatial learning impairment in aged rats that were previously unimpaired in spatial learning tasks. Additionally, anesthesia induced elevated levels of N-methyl-D-aspartate (NMDA) receptor NR2B subunit protein expression in aged. It was concluded that various factors contribute to age-related spatial impairment including: NLRP1 inflammasome activation and NMDA...
receptor NR2B protein expression elevation. It was also concluded that anesthesia exposure exacerbates the elevation in NR2B protein expression in the aged brain, with subsequent disruption of ERK activation leading to chronic spatial learning deficits in aged rats. In the final chapter, a relationship for the interplay between inflammation and NMDA receptor function in the aged brain is discussed. In addition, a novel mechanism for anesthesia-induced cognitive deficits is presented. Therapeutic treatments for cognitive decline and anesthesia-induced cognitive deficits are explored. Finally, future lines of research are proposed.
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<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetyl choline</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid beta precursor protein</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a caspase-activating recruitment domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca²⁺-calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CERAD</td>
<td>Consortium to establish a registry for Alzheimer’s disease</td>
</tr>
<tr>
<td>Ch</td>
<td>Choline</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal regulated protein kinase</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GluR1</td>
<td>Glutamate receptor 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebral ventricular</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<tr>
<td>IL-18</td>
<td>Interleukin 18</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin 1 receptor</td>
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<tr>
<td>IL-1ra</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<td>LTD</td>
<td>Long term depression</td>
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<td>LTP</td>
<td>Long term potentiation</td>
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<td>LRRs</td>
<td>Leucine-rich receptors</td>
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<td>LPS</td>
<td>Lypopolysaccharide</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<td>Mg²⁺</td>
<td>Magnesium ion</td>
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<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>N₂O</td>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal specific nuclear protein</td>
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<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
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<td>NLRP1</td>
<td>NAcht leucine-rich-repeat protein 1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>P2X7</td>
<td>Purinergic receptor P2X ligand-gated ion channel 7</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>POCD</td>
<td>Postoperative cognitive dysfunction</td>
</tr>
<tr>
<td>PPR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PYK2</td>
<td>Proline-rich tyrosine kinase 2</td>
</tr>
<tr>
<td>RAM</td>
<td>Radial arm maze</td>
</tr>
<tr>
<td>Ras-GRF</td>
<td>Ras-specific guanine nucleotide-releasing factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>synGAP</td>
<td>Synaptic Ras GTPase activating protein</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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Chapter I

INTRODUCTION

Anesthesia-related cognitive decline

Normal aging, accompanied by a natural decline in physical and mental capacities, changes the brain’s ability to cope with intrinsic and extrinsic stressors. As a result, the aged brain is distinct in structure and function compared to brains of younger people. Age-related investigations of the brain are especially important due to changes in population growth. People over 65 represent the fastest growing segment of the U.S. population, and they are expected to increase to 53.2% by 2020 (Anderson et al., 2002). Each year, over 21% of people in this age group undergo operations requiring general anesthesia (Ergina et al., 1993). This elderly surgical population faces an increased risk of cognitive complications following surgery that may be due to vulnerabilities in the brain acquired during normal aging.

Postoperative cognitive dysfunction (POCD), defined as “deterioration of intellectual function presenting as impaired memory or concentration” determined by preoperative and postoperative testing (Moller et al., 1998; Rasmussen, 2006), affects 15-25% of surgical patients over 60 years of age (Newman et al., 2007). In fact, age represents the greatest risk factor for development of POCD (Biedler et al., 1999; Moller et al., 2008; Monk et al. 2008; Rasmussen et al., 2001; Steinmetz et al., 2009). POCD, distinct from perioperative delirium that subsides shortly after anesthesia has cleared the body, can persist for weeks, months or even years following surgery (Monk et al., 2008). Cognitive dysfunction after surgery is associated with several negative outcomes including: increased mortality (Monk et al., 2008), risk of leaving the labor market
prematurely, and dependency on social transfer payments (Moller et al., 2008; Steinmetz et al., 2009). Patients with POCD continue to deteriorate following anesthesia for years after surgery generating problems for their families and creating additional drain on the overburdened U.S. healthcare and financial systems.

Clinical studies directed toward this problem are limited due to patient heterogeneity, including age, type of surgery, type of anesthesia, and overall health of patients. Although, animal research has yielded insights into the disorder, mechanisms that contribute to cognitive dysfunction following anesthetic exposure remain unclear. A mechanistic investigation into anesthesia-related cognitive problems is complicated by age-related vulnerabilities in the brain, such as an elevated inflammatory profile of the aged brain. Additionally, age-related changes in N-methyl-D-aspartate (NMDA) receptors, critical in learning and memory, provide a complication in a study of the interaction between age and anesthesia. Therefore, researchers studying underlying mechanisms of POCD must consider age-related changes in the brain involving vulnerabilities, which impart susceptibility of learning and memory problems in the aged brain following anesthesia.

**Age-related cognitive decline**

Aging produces various changes in the brain, including a progressive decline in cognition, particularly in learning and memory processes. The age-related decline in memory performance is closely related to structural and functional changes in the hippocampus (Rosenzweig and Barnes, 2003). The hippocampus demonstrates age-related structural and physiological changes earlier than other brain regions (Driscoll et al., 2009; Kennedy et al., 2009; Raz et al., 2005) including reduction in hippocampal
volume (West, 1993), and a loss in synaptic connections within hippocampal regions (Barnes and McNaughton, 1980; Foster et al., 1991; Geinisman et al., 1992; 2004). Overall, there are significant age-related structural changes within the hippocampus that may impact age-related cognitive decline. Therefore, the hippocampus is an ideal structure to examine in a study of age-related cognitive deficits.

Age-related impairments in learning and memory may involve changes in synaptic connections, neurotransmitters and cytokines (Nieto-Sampedro and Nieto-Diaz, 2005) in the hippocampus. Changes in synaptic plasticity in the hippocampus involving long term potentiation (LTP) have been targeted in the aging literature. LTP, the enhancement of signal transmission between two neurons resulting from simultaneous stimulation, is the cellular mechanism of synaptic plasticity and learning in the hippocampus. Aging can have deleterious effects on LTP under certain conditions. LTP induction does not seem to be adversely affected by age in the presence of high-intensity stimulation in various hippocampal regions (Landfield and Lynch, 1977; Landfield et al., 1978; Barnes, 1979; Diana et al., 1994). However, aging produces deficits in LTP induction when low-intensity stimulation protocols are used (Tielen et al., 1983; Lynch and Voss, 1994; McGahon et al., 1997; Murray and Lynch, 1998). Age-related effects on LTP maintenance are also complicated by experimental protocols. Experiments investigating long periods of LTP maintenance demonstrate deficits (Barnes, 1979), while shorter time protocols do not (Landfield and Lynch, 1977; Diana et al., 1994). The two conditions that seem to be important in determining the age-related effects on LTP are the intensity of the stimuli and the time when LTP maintenance is measured.
Much of the research studying LTP has focused on the NMDA receptor and its function in synaptic plasticity. The NMDA receptor is a ligand-gated cation channel selective for glutamate. Figure 1.1 illustrates a model for involvement of the NMDA receptor during induction of LTP. Briefly, when the NMDA receptor is open, it allows calcium (Ca$^{2+}$) ions into the postsynaptic cell. The NMDA receptor requires concurrent depolarization and glutamate binding to open. However, in the absence of depolarization, the channel is blocked by a magnesium (Mg$^{2+}$) ion, which allows no Ca$^{2+}$ into the cell. Upon simultaneous depolarization and glutamate binding, the channel opens to allow Ca$^{2+}$ into the cell. The Ca$^{2+}$ influx initiates a cascade that ultimately results in LTP (reviewed in Bliss and Collingridge, 1993).

The role of NMDA receptors in spatial learning and memory impairments in aged animals has been studied using Morris water maze (MWM) spatial learning tasks (Morris et al., 1986; Morris, 1989; Morris and Davis, 1994, Gallagher et al., 1993, Magnusson et al., 1997). Figure 1.2 diagrams the water maze apparatus and shows examples of learning versus learning impairment. In the spatial learning navigation task, a rat (or mouse) is placed in a pool of opaque water, with a platform hidden just under the surface of the water. The platform is placed in the same location for all trials, and rodents are released from different points on subsequent trials. In order to escape the water, the rodent must locate the platform, using visual cues, placed around the room. Once learning has occurred, the animals will navigate directly to the platform using these visual spatial cues. Latency to the platform and mean path length are typically used as outcome measures. Aged rats consistently demonstrate spatial learning impairment on this task (reviewed in Rosenzweig and Barnes, 2003). The age-related impairment in spatial
performance in rats on this task mimics learning and memory deficits in aged humans and is often used for investigations of NMDA receptor involvement in rodent models of cognitive impairment.

**The NMDA receptor**

NMDA receptors are a subtype of glutamate receptors, expressed in cortical and hippocampal brain regions, that play a critical role in learning and memory signaling pathways. NMDA receptors are involved in various learning and memory processes and implicated in LTP, the cellular mechanism believed to be involved in memory formation and maintenance (Mondadori *et al.*, 1989; Morris and Davis, 1994; Lisman *et al.*, 1998). NMDA receptors are also required for spatial learning in the Morris water maze (Morris *et al.*, 1986; Morris, 1989; Heal and Harley, 1990; Gallagher *et al.*, 1993; Morris and Davis, 1994; Steel and Morris, 1999). Therefore, NMDA receptors and deficits associated with NMDA receptor signaling in the hippocampus provide an ideal target to study in cognitive deficits associated with aging.

**Subunits and neuronal plasticity:** NMDA receptors are tetrameric complexes made up of two NR1 and two NR2 subunits. The different NR2 subunits (NR2A-NR2D) confer distinct gating and pharmacological properties on the receptor channel. NR2A and NR2B are the predominate NR2 subunits in the hippocampus (Sheng *et al.*, 1994). Most NR2A-containing NMDA receptors are incorporated into synapses. NR2B-containing NMDA receptors are also present in synapses, but they are mainly found extrasynaptically (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Liu *et al.*, 2004). Subunit composition of NMDA receptors can have differential effects on downstream signaling molecules, such as the extracellular-signal regulated protein kinase
(ERK) pathway. Due to the requirement of ERK activation for spatial learning acquisition (Atkins et al., 1998; Selcher et al., 1999), NMDA receptor subunit composition and localization has specific consequences for behavioral outcomes. NR2A is coupled to the activation of ERK, while NR2B is coupled to ERK inhibition (Kim et al., 2005). Additionally, the location of NMDA receptors, whether synaptic or extrasynaptic, has differential effects on ERK activation (Hardingham, 2006). Figure 1.3 shows the opposite effects on ERK1/2 when synaptic versus extrasynaptic NMDA receptors are activated. Taken together, it is possible that activation of synaptic NR2A-containing NMDA receptors enhances spatial learning, while activation of extrasynaptic NR2B-containing NMDA receptors inhibits spatial learning. Furthermore, NMDA receptor activation has differential effects on α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor trafficking, depending on the subunit composition of NMDA receptors, such that NR2A-NMDA receptors promote, whereas NR2B-NMDA receptors inhibit the surface expression of glutamate receptor 1 (GluR1), by regulating its surface insertion (Kim et al, 2005). GluR1 surface expression has been implicated in activity-dependent synaptic plasticity (MacDonald et al., 2006). Taken together, NMDA receptor activation produces functional consequences in learning and memory signaling and behavioral outcomes depending on the ratio of NR2A to NR2B in synaptic and whole brain regions.

The effects of aging are not equal across brain regions or NMDA receptor subunits. NMDA receptors seem to have greater vulnerability to normal aging than other glutamate receptors. These receptors demonstrate age-related declines in binding strength and protein expression changes. Age-related changes in NMDA receptors correspond to
age-related cognitive decline. However, recent evidence indicates that among older individuals, those with the highest level of protein expression demonstrate poor learning performance, suggesting a functional change in the NMDA receptor during normal aging (Zhao et al., 2009).

**Effect of aging on receptor binding:** Binding studies of aging and the NMDA receptor reveal a decrease in binding affinity of agonists and antagonists in the cortex and hippocampus of aged animals (Ammassasri-Teule and Gozzo, 1982; Eichenbaum et al., 1990). The decline in NMDA receptor binding affinity has been reported in several mammalian species, such as Fischer 344 (Castorina et al., 1994; Clark et al., 1992; Davis et al., 1993; Ingram et al., 1992; Kito et al., 1990; Ogawa et al., 1992; Tamaru et al., 1991), Sprague-Dawley (Fiore and Rampello, 1989) Long Evans (Peleymounter et al., 1990), and Wistar (Serra et al., 1994) rats, C57BL/6 and BALB/c mice (Magnusson and Cotman, 1993; Magnusson et al., 1995; 1997; 2000), dogs (Magnusson et al., 2000) and rhesus monkeys (Wenk et al., 1991). Although declines in binding strength are consistently observed in aged animals, the age-related decline in NMDA binding is not homogenous across brain regions. The cortex seems to show greater decreases in binding than the hippocampus (Castorina et al., 1994; Davis et al., 1993; Magnusson, 1995; 2001 Magnusson and Cotman, 1993; Tamaru et al., 1991). The binding site changes within the hippocampus also vary across regions. NMDA receptor binding sites in the dorsal hippocampus are less susceptible to age-related changes than those observed in the intermediate hippocampus in mice (Magnusson et al., 2006). Reports of variability within the hippocampus are also demonstrated by differences between NMDA receptor binding in the intermediate hippocampus of mice (Magnusson and Cotman, 1993; Magnusson,

The variability of the effects of aging on NMDA receptor binding suggests there are changes in the remaining receptors with normal aging. The differences in susceptibility to aging of NMDA receptor binding within different brain regions may be due to the differing populations of subunits, or a vulnerability of some regions to age-related oxidative damage. Evidence to support the latter view involves an increase in oxidation of the redox site on NMDA receptors in aged rats versus young rats (Bodhinathan et al., 2007). In addition, it is likely that protein expression changes and functional consequences of such changes contribute to age-related declines in memory.

*Age-related changes in protein expression:* The effects of aging on NR1 protein expression are variable between species. Various researchers have demonstrated decreases in protein expression of NR1 in the hippocampus and cortex of aged mice (Magnusson et al., 2002), Fischer rats (Coultrap et al., 2008; Eckles-Smith et al., 2000; Mesches et al., 2004; Newton et al., 2007; Shi et al., 2007), and macaque monkeys (Gassaley et al., 1996). However, in Wistar and Long-Evans rats, no age-related decline in protein expression of NR1 subunit was reported (Adams et al., 2001; Dyall et al., 2007). Experience can have differential effects on NR1 protein expression. Experiences such as performing tasks involving reference memory, working memory, associative memory, and spatial learning memory can lead to an increase in NR1 protein expression in the hippocampus (Das and Magnusson, 2008). LTP also increases NR1 subunit protein expression in rats (Clayton et al., 2002). Therefore, the variability of the age-related protein expression decreases in NR1 within species and between strains may indicate a
role for environmental factors during aging, instead of a genetic program for age-related
decline in NR1 subunit protein expression.

Age seems to have little effect on NR2A protein expression in the brain. NR2A protein expression changes were not detected in the hippocampus of aged Fischer rats (Clayton and Browning, 2001; Clayton et al., 2002; Mesches et al., 2004), F344XBN rats (Newton et al., 2007), or the striatum of Wistar rats (Martinez-Villayandre et al., 2006). There is a reported decline in NR2A protein expression in the hippocampus of C57BL/6 mice that occurs between middle-age and old-age (Magnusson et al., 2002). Also, female, Sprague-Dawley rats show less decline in protein expression of the NR2A subunit compared to declines in NR1 and NR2B in the hippocampus (Adams et al., 2001), while males show no change in the subunit (Delibas et al., 2005). Overall, the NR2A subunit is less susceptible to age-related changes than the other subunits.

Evidence for declines in protein expression of the NR2B subunit demonstrate that NR2B has the greatest susceptibility to aging. Age-related declines in protein expression of NR2B are greater than those observed for NR1 in the hippocampus and cortex of aged C57BL/6 mice (Magnusson et al., 2000; 2002). Additionally, there may be an effect of aging on NR2B protein expression in synaptic membranes in the cortex of mice. Zhao and colleagues (2009) demonstrated that the decline of NR2B protein expression was greater in the synaptic membrane fraction than the whole homogenate. Changes in the synaptic membrane protein expression of NR2B subunit may be associated with changes in subunit composition at the synapse, contributing to changes in function of NMDA receptors and the interaction of these receptors and other proteins involved in synaptic plasticity. In the hippocampus, the whole homogenate and synaptic membrane fraction
demonstrated equal age–related decline in NR2B expression (Zhao et al., 2009). Together, these results indicate that protein expression of the NR2B subunit is significantly decreased in the cortex and hippocampus of aged animals.

In addition to evidence suggesting age-related declines in NMDA receptor binding densities and subunit expression associated with declines in learning and memory performance, there is evidence indicating a functional change in the remaining NMDA receptor subunits in the aged brain. High levels of NR1 and NR2B in the brains of aged mice were found to be correlated with the poorest performance on spatial memory tasks (Zhao et al., 2009). High densities of NMDA receptor binding in the hippocampus of aged rats were associated with poor long-term memory retention in Morris water maze (Topic et al., 2007). Additionally, inhibition of NMDA receptors by Memantine (a drug used to treat Alzheimer’s disease (AD)) promotes spatial learning improvements in aged animals (Beracochea et al., 2008; Chen et al., 2008; Danysz and Parsons, 2003; Pieta Dias et al., 2007). These results are counterintuitive when considering the importance of these receptors in acquisition of spatial learning (Heale and Harley, 1990; Morris, 1989; Steele and Morris, 1999), suggesting age-related changes in the function of NMDA receptors containing NR1 and NR2B subunits in the synaptic membrane. This change may be associated with compensatory mechanisms related to the loss of receptors due to aging. Additionally, aging may affect other learning and memory proteins that interact with NMDA receptors.

**Inflammation in the aged brain**

Normal aging also involves an increase in inflammatory processes in the central nervous system (CNS). Age-related inflammation is associated with several
neurodegenerative diseases such as AD, Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), the Parkinson-dementia complex of Guam, all the tauopathies, and age-related macular degeneration. Even in the absence of neurological disease, the aged brain exhibits a heightened inflammatory profile (Lee et al., 2000), with a decrease in the production of anti-inflammatory cytokines (Straub et al., 2000), and an increase in pro-inflammatory cytokines (Godbout and Johnson, 2004). Consequently, the elevated inflammatory profile in the elderly brain may contribute to cognitive problems related to aging.

Evidence suggests that age is a risk factor for delirium and dementia following an infection. Clinically, cognitive and behavioral disorders are more frequently reported in elderly patients with an infection or illness (Jackson et al., 2004; Mulsant et al., 1999). Delirium, a risk factor for dementia (Gillis et al., 2006; Jitapunkel et al., 1992; Lin et al., 2010; Margiotta et al., 2006; O’Brien et al., 1992), is often associated with infection (Jackson et al., 2004; Janssens and Krauss, 2004; Rockwood et al., 1999). Elderly patients with prolonged infections also had an increased probability of developing dementia (George et al., 1997) and increased mortality rates (Rockwood et al., 1999). A heightened inflammatory profile exposes the aged brain to an increased release of inflammatory cytokines, in response to extrinsic stressors. The heightened inflammatory profile of the aged brain may lead to cognitive impairments as a result of prolonged exposure to inflammatory cytokines.

Activation of the inflammatory response involves the release of inflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNFα). These cytokines communicate activation of the inflammatory response to the brain, by
activating microglia. Microglia, the resident innate immune cells of the brain, induce and propagate these cytokines throughout the CNS (Rivest, 2003). Microglia, quiescent in the absence of stimulation (Gonzalez-Scarano and Baltuch, 1999), modulate neuroinflammatory signals in the brain. Various researchers have demonstrated an increase in glial reactivity in the aged brain (reviewed Godbout and Johnson, 2009). The age-related elevation in the inflammatory profile involving increased glial reactivity (Frank et al., 2005; Godbout et al., 2005; Morgan et al., 1999; Perry et al., 1993) may initiate an exaggerated neuroinflammatory cytokine response in the aged brain. The heightened innate inflammatory response in the aged brain may have deleterious consequences on cognitive behavioral outcomes, manifesting as age-related cognitive decline.

Inflammatory cytokines may play a role in age-related cognitive deficits by disrupting synaptic plasticity. Prolonged exposure to IL-1β may disrupt neuronal plasticity (Godbout and Johnson, 2009), and is associated with cognitive dysfunction (Godbout et al., 2005; Heyser et al., 1997; Monje et al., 2003; Vallieres et al., 2002; Vereker et al., 2000). Inflammatory cytokines disrupt neuronal functions involved in synaptic plasticity, such as LTP, neurite outgrowth, and neurogenesis. Many researchers have demonstrated that inflammatory cytokines disrupt LTP in the hippocampus (Lie et al., 1997; Lynch et al., 2004; Vereker et al., 2000). One study showed that treating rat hippocampal brain slices with IL-6 abolished LTP (Li et al., 1997). Peripheral injection with lipopolysaccharide (LPS) also impaired LTP in the rat hippocampus via increases in IL-1β production (Vereker et al., 2000). Recombinant IL-1β, administered by intracerebral ventricular (ICV) infusion, reduced hippocampal LTP (Vereker et al.,
Moreover, the reversal of LTP inhibition was demonstrated by ICV infusion of the anti-inflammatory cytokine, IL-10 (Lynch et al., 2004). Neurite outgrowth and branching, an important process by which existing synaptic connections are modified, was also inhibited by IL-1β in hippocampal cultures via TNFα production (Neumann et al., 2002). Neurogenesis, critical in learning and memory process, is also disrupted by inflammatory cytokines. Neuroinflammation, induced by ICV or intraperitoneal (IP) injection of LPS, reduced hippocampal neurogenesis (Monje et al., 2003), while anti-inflammatory drugs, idomethacin and minocycline, restored neurogenesis (Ekdahl et al., 2003). Taken together, cytokines involved in neuroinflammation disrupt synaptic plasticity that may underlie some age-related cognitive problems.

Various cellular and molecular inflammatory response mechanisms contribute to normal age-related cognitive decline, including increased glia reactivity, and elevated levels of inflammatory cytokines. These mechanisms may be non-specific cellular events that are part of the progression of normal aging. In consideration of the elevated inflammatory profile and response in the aged brain, it is possible that other inflammatory processes may also contribute to the elevated inflammatory cytokine profile in the aged brain. One such neuronal inflammatory response that has not yet been investigated in the aged brain is the NLRP1 inflammasome.

The NLRP1 Inflammasome

The NLRP1 inflammasome, a multiprotein complex consisting of caspase-1, caspase-11, apoptosis-associated speck-like protein containing a caspase-activating recruitment domain (ASC), and NAcht leucine-rich-repeat protein 1 (NLRP1) is expressed in neurons (de Rivero Vaccari et al., 2008). The NLRP1 inflammasome forms
a protein assembly with the X-linked inhibitor of apoptosis protein (XIAP). The NLRP1 inflammasome is activated by recognition of pathogen-associated molecular patterns (PAMPs). Specifically, the recognition of PAMPs by a membrane-type or cytosolic pattern recognition receptor (PPR) family of cytosolic leucine-rich receptors (LRRs) called nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) occurs in the NLRP1 inflammasome. Stimulation of the inflammasome complex activates an inflammatory response via processing of proinflammatory cytokines, IL-1β and IL-18 (Martinon et al., 2002; Martinon and Tschopp, 2004; Miao et al., 2006; Ozoren et al., 2006). IL-1β is produced and stored in the cytosol in an inactive form, pro-IL-1β. The inactive form is rapidly cleaved by caspase-1 upon activation (Thornberry et al., 1995). The active IL-1β is released from the cell activating inflammation. Activation of the NLRP1 inflammasome in neurons has recently been shown to play a critical role in the inflammatory response to spinal cord injury (de Rivero Vaccari et al., 2008) and stroke (Abulafia et al., 2009). However, the involvement of the inflammasome complex in the aged brain remains unclear. Based on the extensive literature implicating inflammation in age-related cognitive decline, it is possible that the inflammasome plays a critical role in the inflammatory response and cognitive impairment the aged brain.

**Link between inflammation and POCD**

A specific relationship between anesthesia and inflammation was recently reported. Wu and colleagues (2010) demonstrated that isoflurane anesthesia induced an increase in mRNA and protein levels of pro-inflammatory cytokines TNFα, IL-6, and IL-1β in the brains of young mice. Additionally, amounts of TNFα immunostaining in the brain tissues were elevated. Isoflurane also induced an increase in TNFα protein levels in
primary neurons. These results demonstrate the potentially neurotoxic effects of isoflurane mediated through inflammatory processes.

It has also been suggested that the combination of surgical trauma, anesthesia, and stress induces a nonspecific inflammatory response during surgery. After major surgery, the main inflammatory cytokines released systemically are IL-1β, TNFα, and IL-6. Elevated concentrations of the pro-inflammatory cytokine, IL-6, were present in cerebrospinal fluid samples from surgical patients 1 week after coronary bypass grafting surgery, with an increased concentration of IL-4, an anti-inflammatory cytokine, 6 months after surgery (Kalman et al., 2006). Inflammatory cytokines can mediate neuronal plasticity, influencing cognitive decline. Therefore, a non-specific inflammatory response may contribute to POCD. The elevated inflammatory response in the aged brain contributes to the susceptibility of cognitive dysfunction in the elderly population, and also imparts susceptibility of extrinsic stressors and trauma, such as surgery with anesthesia, which may be exacerbated in the aged brain.

Anesthesia and the aged brain

Clinical studies of POCD: In 1955, prompted by reports from elderly surgical patients and their families, Bedford conducted a retrospective investigation into the manifestation of cognitive dysfunction in older patients following general anesthesia. He concluded that older patients that received general anesthesia had a significantly greater risk of developing such deficits than their younger surgical counterparts. The alteration in cognitive function following anesthesia later became known as POCD.

More extensive clinical studies have been conducted to evaluate POCD in elderly surgical populations. However, the incidence of POCD remains controversial. One study
reported a staggering 90% of elderly patients developing symptoms of POCD (Ancelin et al., 2001), while others report no incidence of anesthesia-related cognitive decline (Chung et al., 1990; Giberstadt et al., 1968; Goldstein et al., 1993; 1998) and some even report an improvement in memory tasks following surgery (Billig et al., 1996; Elam et al., 1988; Hall et al., 2005). Table 1.1. describes several clinical studies aimed at determining the incidence of POCD in surgical populations.

The existing controversy regarding the incidence of POCD exists mainly due to a lack of standardized methodology to identify and test for anesthesia related cognitive dysfunction. Newman and colleagues (2007) pointed out several limitations in their review of over 40 clinical studies. Some of the methodological problems in the literature include: no age-matched controls used to compare to surgical patients, and no standardized statistical methods to analyze the data. Also, many studies were underpowered. The definition of POCD also seems to be an overwhelming problem in the literature, when comparing across clinical studies. Several other variables are present between studies, including: the use of different neuropsychological tests, timing of testing, amount of significant change and exclusion criteria. Many of the tests used are also used to identify dementia. Therefore, it is difficult to define the presence of POCD separately from other dementia-type disorders. Commonly used tests for the presence of POCD include: the Logical Memory Test, the Consortium to establish a registry for Alzheimer’s disease (CERAD) word list memory, the Boston naming test, Category Fluency test, Digit Span Test, Trail making test and Digit symbol substitution test (Bryson et al., 2006; Conhendy et al., 2005; Lombard et al., 2010; Rasmussen et al., 2001; Rubens et al., 2007). Researchers typically use a battery of tests to determine
anesthesia-induced deficits. Verbal learning, working memory, episodic memory, processing speed, and set shifting demonstrate the greatest sensitivity to cognitive decline following surgery (Newman et al., 2007). However, scoring methods can mask the differences between groups. Floor effects and an effect of individuals with low baseline scores requiring minimal change in their raw score to be categorized POCD sufferers are also limitations of scoring methods (Deiner and Silverstein, 2009). Time of testing after exposure to anesthesia is also important. Significant variability between testing sessions for individuals has been reported due to learning and examiner bias (Laursen et al., 1997). Also, patients may experience anxiety about surgery and perform poorly just before surgery as a result. The time in which testing is performed after surgery may also affect performance. Immediately after an operation, performance can be influenced by pain, medications, and incomplete recovery from surgery. Long-term postoperative testing is confounded by attrition. It is possible that those patients most affected by cognitive decline following surgery may drop out of the study. Therefore, it is likely that some reports have underestimated the prevalence of the disorder.

Despite controversy in the incidence of POCD and the methodological limitations in the literature, clinical studies have identified several risk factors associated with development of anesthesia-related cognitive decline. Monk and colleagues (2008) conducted a study of 1064 surgical patients from three age groups: 117 young (18-39 years), 112 middle-aged (40-59 years) and 138 elderly (60+ years). They found that POCD was present at hospital discharge in 36.6% of young, 30.4% of middle-aged, and 41.4% of elderly patients. At 3 months post-anesthesia, the presence of POCD was similar between young and middle-aged patients to age-matched controls. However, the
elderly group demonstrated a higher incidence of POCD (12%) than controls. They also identified independent risk factors for development of POCD at 3 months post-surgery, including: advanced age, lower educational level, history of previous cerebrovascular disease/incident, and the presence of POCD at hospital discharge. Longitudinal studies conducted in Europe also found age to be a predictor of POCD (Moller et al., 2008; Steinmetz et al., 2009). Duration of anesthesia, intra-operative complications and postoperative infections were also significant factors involved in the development of POCD (Biedler et al., 1999; Rasmussen et al., 2001). Researchers have identified postoperative delirium as a strong predictor for development of subsequent chronic cognitive dysfunction in the elderly (Bickel et al., 2008; Kat et al., 2008; Rudolph et al., 2008). Other risk factors include: major surgery (Canet et al., 2003; Rasmussen et al., 2004), history of alcohol abuse (Hudetz et al., 2007), change in thyroid hormone postoperatively (Mafrika et al., 2008), cerebral trauma (Goto et al., 2001; Tan et al., 2008), and preoperative cognitive decline (Ancelin et al., 2001). Interestingly, depth of anesthesia (Farrag et al., 2006; Steinmetz et al., 2010) and general versus regional anesthesia (Bryson et al., 2006; Rasmussen et al., 2003; Wu et al., 2004) were not correlated with development of cognitive decline following surgery. Taken together, there are several risk factors that increase the likelihood of developing POCD, with advanced age representing the greatest risk factor in surgical patients.

In summary, the clinical studies that investigated POCD in the elderly provide awareness about the prevalence of anesthesia-related cognitive decline, despite controversy over the percentage of elderly surgical patients affected. They also identify risk factors associated with development of POCD. However, the methodological
variability between studies prevents researchers from drawing conclusions about causality of cognitive decline following surgery and anesthetic exposure. Several questions remain unanswered: does anesthesia hasten the progression of normal aging; are some elderly individuals more susceptible to the detrimental effects of anesthesia; are there specific biochemical pathways that are disrupted by anesthesia in the aged brain, and if so, do they represent targets for therapeutic intervention? Studies using both in vitro cultures systems and in vivo rodent models may provide insight into such questions, providing some explanation of anesthesia-related cognitive decline in the elderly.

*The effects of isoflurane in vitro:* The toxic effects of isoflurane anesthesia are evident in various culture systems, where exposure results in cell damage and apoptotic cell death. Zhen and colleagues (2009) demonstrated the toxicity of isoflurane/nitrous oxide mixture by exposing H4 (human neuroglioma 4) naïve cells to the anesthetics. The mixture promoted toxicity by inducing apoptosis via caspase 3 activation. In chicken B-lymphocytes, isoflurane induced concentration-dependent and time-dependent apoptosis, and elevated cytosolic and mitochondrial calcium levels (Wei et al., 2008). Additionally, isoflurane exposure of H4 cells over-expressing amyloid precursor protein (H4-APP) demonstrated increased levels of amyloid beta (Aβ) (Eckenhoff et al., 2004; Xie et al., 2006). Increases in the accumulation and aggregation of Aβ following isoflurane were also reported (Xie et al., 2007; Zhen et al., 2009; Perucho et al., 2010), linking isoflurane to AD pathology.

Evidence for the effects of isoflurane on neuronal function has also been conducted on in vitro experiments, demonstrating the inherent neurotoxicity of the anesthetic. Primary neuron cultures from mice exposed to isoflurane increased cleavage
of caspase 3, a marker of apoptosis (Head et al., 2009; Zhen et al., 2009). Another study demonstrated isoflurane-induced RhoA activation, cytoskeletal depolymerization and apoptosis in primary neuron cultures (Lemkull et al., 2011). In primary rat cortical neurons, Wang and colleagues (2008) demonstrated isoflurane-induced cytotoxicity with an increase in intracellular calcium concentrations partially through elevated activity of IP3 receptors. Thus, isoflurane anesthesia may produce specific cellular consequences in the CNS, resulting in cellular damage and death. The previous experiments provided valuable insight into the effects of isoflurane on neurons, yet these studies were unable to mimic the anesthetic conditions experienced by the CNS. Animal models of POCD provide a useful tool to study the extensive effects of general anesthesia on the CNS and investigate anesthesia-related cognitive dysfunction in the whole organism.

Animal models of anesthesia-related cognitive dysfunction: Animal studies implicate isoflurane, a commonly used anesthetic, in neurotoxicity and learning and memory impairments in vivo. In the developing brain, isoflurane exposure produces widespread neuronal cell death (Brambrink et al., 2010; Istaphanous and Loepke, 2009; Jevtovic-Todorovic et al., 2003; Johnson et al., 2008; Nikizad et al., 2007Wu et al., 2010). Brambrink and colleagues (2010) investigated the effect of isoflurane exposure in rhesus monkeys (postnatal day 6), demonstrating extensive neuronal apoptosis in cortical regions (Brambrink et al., 2010). Jevtovic-Todorovic and colleagues (2003) reported widespread cell death in the infant rat brain following exposure to midazolam, nitrous oxide and isoflurane. When the rats were behaviorally tested, those exposed to all three anesthetics demonstrated severe learning and memory impairments. These results indicate that isoflurane produces extensive neurodegeneration in the developing brain.
The adult brain also exhibits vulnerabilities to isoflurane anesthetic exposure, resulting in behavioral impairments. Using in vivo microdialysis techniques in freely moving rats, Wang and colleagues (2009) demonstrated increased release of Acetyl choline (Ach) and choline (Ch) in aged rats (18 months) that displayed post-anesthetic spatial learning impairments in Morris watermaze (MWM). Taken together, two distinct impairment groups in aged rats following anesthetic insult were identified: “cognition-markedly-impaired” and “cognition- lightly-impaired”, in which the hippocampal release of Ach and Ch were significantly different between the two groups (Wang et al., 2009). This study suggests that certain individuals may have previously unappreciated pre-existing conditions that impart greater vulnerability to the deleterious effects of anesthesia.

Age at the time of exposure, in part, determines the effect of anesthesia on cognitive function. Isoflurane-induced improvements in spatial memory tasks were reported in young rats (Culley et al, 2003, 2004) and mice (Rammes et al, 2009). Rammes and colleagues (2009) exposed male C57BL6/J adult (4-5 months) mice to isoflurane anesthesia for 2 hours, then evaluated neurocognitive function, LTP in vitro, and protein expression at 24 hours post-anesthesia. Anesthetized mice demonstrated improved cognitive performance. In hippocampal slice preparations of anesthetized mice, LTP was enhanced. Protein expression analysis revealed an upregulation of NR2B in hippocampal neurons after anesthesia. The results of this study indicate that young rats show improvements in spatial learning via an increase in hippocampal-specific NR2B protein expression, and enhanced LTP in CA1 neurons. In contrast, aged (18-20 months) rats experience isoflurane-induced impairments in spatial memory task up to 2 months
post-anesthesia (Culley et al., 2003; 2004). Culley and colleagues (2003) were the first to demonstrate age-specific impairments in rodents following anesthetic exposure. In that study, young (6 month) and aged (18 month) Fischer 344 rats were tested in a 12-arm radial arm mask (RAM) task. Working and reference memory were assessed. Following baseline testing, rats were assigned to receive 2 hours of isoflurane anesthesia or no anesthesia. Rats were again tested in RAM at 1, 3, and 8 weeks post-anesthesia. Anesthesia-treated aged rats demonstrated sustained learning impairments compared to controls, while young rats demonstrated improvement regardless of anesthesia treatment (Culley et al., 2003). A follow-up study demonstrated impairment in spatial acquisition memory in aged (18 month) rats two weeks after anesthetic exposure (Culley et al., 2004). The cognitive problems induced by general anesthesia did not reduce life expectancy in older rats (Culley et al, 2005), but mimicked some of the impairments reported by patients suffering from POCD. Age may represent a significant risk factor for anesthesia-induced cognitive impairments in rodents similar to the age-related risk observed in humans. The biochemical mechanisms by which isoflurane produces poor cognitive performance in aged, but not young, rats remains unknown.

**Research objective and hypothesis**

Cognitive decline following anesthetic exposure in elderly surgical patients represents an increasingly prevalent problem. Although evidence has suggested potential mechanisms of POCD involving inflammation, anesthetic neurotoxicity, and the role of NMDA receptors in age-related cognitive dysfunction, a mechanism for the interaction between advanced age and anesthesia remains lacking. It is expected that the elucidation of specific mechanisms of POCD will provide therapeutic targets for attenuation and
prevention of anesthesia-associated cognitive problems in the growing elderly surgical population. My findings will advance the fields of anesthesia, age-associated disorders, and age-specific inflammation, with specific contributions to studies of the interaction between normal aging and CNS insults, such as anesthesia.

The goal of this thesis is to investigate the effect of isoflurane anesthesia on spatial learning involving NMDA receptor signaling and downstream learning and memory pathways (i.e. ERK1/2) in the aged brain. I hypothesize that isoflurane anesthesia induces an increase in protein expression of NMDA receptor subunit NR2B, corresponding to decreased activation of ERK1/2. The detrimental behavioral outcome of these molecular changes is discussed. Also, it is important to identify vulnerabilities present in the aged brain that interact with anesthesia exposure, specifically elevated inflammatory profiles and functional differences in NMDA receptor subunit protein expression that may impact behavioral outcomes for aged animals. It is discussed how the interaction of anesthesia and vulnerabilities associated with normal aging correspond to behavioral outcomes. These studies will establish whether isoflurane affects NMDA receptor mediated deficits in learning and memory pathways. Such information is important because it will allow for the development of pharmacological treatments to prevent cognitive dysfunction in the elderly following exposure to general anesthetics.

In chapter 2, inflammasome activity in the aged brain is investigated. Specifically, an elevation in NLRP1 inflammasome components is linked to spatial learning impairments in aged male Fischer 344 rats.
In chapter 3, NR2B-containing NMDA receptors involvement in isoflurane-induced caspase-3 activation and cell death are investigated. Specifically, NR2B-specific NMDA receptor antagonists attenuate isoflurane-induced cell death.

In chapter 4, anesthesia-induced changes in NMDA receptor subunits in spatially impaired aged rats are investigated. Specifically, an increase in NMDA receptor subunit NR2B in the hippocampus and cortex at acute and chronic time points corresponds to spatial learning deficits in aged male Fischer 344 rats.

In the last chapter, evidence for anesthesia-induced mortality is presented and discussed along with a general discussion and review of the central findings in the thesis. The significance and contributions of the findings to the fields of aging, inflammation, and anesthesia-associated cognitive deficits are discussed. Finally, a discussion of future research directions arising from these findings are presented.
## Tables for Chapter I

### Table 1.1. Incidence of POCD following surgery

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Study size</th>
<th>Type of Surgery</th>
<th>Anes</th>
<th>Age Range, Avg. Age</th>
<th>Testing point</th>
<th>Finding of % decline in surgical group (sig.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1968</td>
<td>Giberstadt</td>
<td>63</td>
<td>Abdominal</td>
<td>GA</td>
<td>54-75, 66.71</td>
<td>6mo-1y</td>
<td>no decline; no decline</td>
</tr>
<tr>
<td>1987</td>
<td>Shaw</td>
<td>48**</td>
<td>Major vascular</td>
<td>GA</td>
<td>41-68, 57.4</td>
<td>7-21 days</td>
<td>31%, controls not compared</td>
</tr>
<tr>
<td>1988</td>
<td>Elam</td>
<td>164*</td>
<td>Cataract</td>
<td>NR</td>
<td>35-85, 60.3</td>
<td>3wks-6mo</td>
<td>No decline (improved)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6mo-1y</td>
<td>No decline; improved</td>
</tr>
<tr>
<td>1989</td>
<td>Treasure</td>
<td>24*</td>
<td>Thoracic, major vascular</td>
<td>GA</td>
<td>22-85, 62</td>
<td>7-21 days</td>
<td>No change</td>
</tr>
<tr>
<td>1990</td>
<td>Chung</td>
<td>40*</td>
<td>Cholecystectomy</td>
<td>GA</td>
<td>55-82, 67.0</td>
<td>3wks-6mo</td>
<td>No change</td>
</tr>
<tr>
<td>1993</td>
<td>Goldstein</td>
<td>62*</td>
<td>General, orthopedic</td>
<td>GA</td>
<td>55-87, 66.5</td>
<td>3wks-6mo</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54*</td>
<td></td>
<td></td>
<td></td>
<td>6mo-1y</td>
<td>No change</td>
</tr>
<tr>
<td>1996</td>
<td>Billig</td>
<td>108</td>
<td>Cataract</td>
<td>GA/LA</td>
<td>NR, 75.9</td>
<td>7-21 days</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3wks-6mo</td>
<td>No change in MMSE, significant improvement in 2 tests 9% (n.s.)</td>
</tr>
<tr>
<td>1998</td>
<td>Goldstein</td>
<td>104</td>
<td>General, orthopedic</td>
<td>GA</td>
<td>NR, 68 (median)</td>
<td>6mo-1y</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td>172</td>
<td></td>
<td></td>
<td></td>
<td>3wks-6mo</td>
<td>No decline</td>
</tr>
<tr>
<td>1998</td>
<td>Moller</td>
<td>1214</td>
<td>Mixed</td>
<td>GA</td>
<td>60-79, 68</td>
<td>7-21 days</td>
<td>25.80% ($P&lt;0.001$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3wks-6mo</td>
<td>9.90% ($P=0.0037$)</td>
</tr>
<tr>
<td>1999</td>
<td>Dijkstra</td>
<td>48</td>
<td>Major mixed</td>
<td>GA</td>
<td>60-85, 68.2</td>
<td>7-21 days</td>
<td>27% ($P=0.048$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>947</td>
<td></td>
<td></td>
<td></td>
<td>3wks-6mo</td>
<td>2% (n.s.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44.80%</td>
</tr>
<tr>
<td>1999</td>
<td>Grichnik</td>
<td>29*</td>
<td>Thoracic, vascular</td>
<td>GA</td>
<td>NR, 53.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>Abildstrom</td>
<td>336</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>Rasmussen</td>
<td>53‡</td>
<td>Abdominal</td>
<td>GA</td>
<td>NR, 75.9</td>
<td>7-21 days</td>
<td>32.70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3wks-6mo</td>
<td>2.8</td>
</tr>
<tr>
<td>2000</td>
<td>Stockton</td>
<td>274*</td>
<td>Cataract</td>
<td>GA/LA</td>
<td>60-93, 73</td>
<td>7-21 days</td>
<td>n.s.</td>
</tr>
<tr>
<td>Year</td>
<td>Name</td>
<td>Speciality</td>
<td>Anes</td>
<td>Age Range</td>
<td>Duration</td>
<td>MMSE Decrease</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
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<td>------</td>
<td>-----------</td>
<td>----------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>Ancelin</td>
<td>Orthopedic general, orthopedic</td>
<td>LA</td>
<td>64-87, 72.6</td>
<td>7-21 days</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2001</td>
<td>Urinary system, genitourinary</td>
<td>GA/EA</td>
<td>64-87, 72.6</td>
<td>3 wks-6 mo</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>Farrag</td>
<td>Gynecology</td>
<td>GA</td>
<td>NR, 41.4</td>
<td>3 wks-6 mo</td>
<td>Significant decline MMSE</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>Johnson</td>
<td>Abdominal, orthopedic</td>
<td>GA</td>
<td>41-59, 50.5</td>
<td>7-21 days</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>Canet</td>
<td>Minor</td>
<td>GA</td>
<td>61-80, 67.7</td>
<td>7-21 days</td>
<td>6.20% (n.s.)</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>Iohom</td>
<td>Abdominal</td>
<td>GA</td>
<td>55-87, 66.5</td>
<td>3 wks-6 mo</td>
<td>6.6 (P=0.03)</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Hall</td>
<td>Cataract</td>
<td>NR</td>
<td>NR, 70.9</td>
<td>6 mo-1 y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Rodriguez</td>
<td>Knee replacement</td>
<td>GA/EA</td>
<td>45-82, 69.0</td>
<td>7-21 days</td>
<td>41%</td>
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<td>2008</td>
<td>Bickel</td>
<td>Orthopedic</td>
<td>GA</td>
<td>Over 60</td>
<td>38 months</td>
<td>10.2%</td>
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<tr>
<td>2008</td>
<td>Monk</td>
<td>Non-cardiac</td>
<td>GA</td>
<td>18-60+, 50.4</td>
<td>Hospital discharge</td>
<td>36% young, 30.4% middle-aged, 41.4% elderly</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>Price</td>
<td>Non-cardiac; minimally invasive, intra-abdominal/thoracic, orthopedic</td>
<td>GA</td>
<td>Over 60, 68.3</td>
<td>Hospital discharge</td>
<td>3 months</td>
<td></td>
</tr>
</tbody>
</table>

*no controls, **data on controls was not compared to patient group, ‡previously gathered controls, Anes=type of anesthesia, GA=general anesthesia, EA=epidural anesthesia, LA=local anesthesia, RA=regional anesthesia, NR=not reported, n.s.=not significant
Figure 1.1 Model for the induction of LTP (modified from Malenka and Nicoll, 1999). During normal synaptic transmission, glutamate (Glu) is released from presynaptic cell and acts on both 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. However, Na\(^+\) flows only through the AMPA receptor, but not the NMDA receptor because Mg\(^{2+}\) blocks the NMDA receptor in the resting state. Depolarization of the postsynaptic cell removes the Mg\(^{2+}\) block, allowing Na\(^+\) and Ca\(^{2+}\) to flow into the postsynaptic cell. The rise in Ca\(^{2+}\) in the postsynaptic cell is the critical trigger for the induction of long term potentiation (LTP).
Figure 1.2 Diagrammatical illustration of Morris water maze testing room and apparatus (modified from Terry, 2009) The left panel shows a large circular pool (180 cm diameter, 76 cm height), filled with water (maintained at 25°C) made opaque with white paint, approximately 35 cm deep to cover a hidden platform, submerged 10 cm below the surface of the water. The pool is located in a large room with visual cues placed on the walls. A camera is affixed directly above the pool, and lights are affixed on the ceiling to illuminate the room. Each day of testing, a trial is initiated by placing the rat in the pool at one of four points on the pool (designated N, S, E, and W). The rat is allowed 60 seconds to find the hidden platform. If the rat fails to find the platform in the time allotted the experimenter places the rat on the platform, where the rat is allowed to remain for 10 seconds. Then the rat is placed back in the home cage. Four trials are conducted each day. The left panel shows an example of the computer software (EthoVision) generated path length track for a single trial. On trial 1, the rat explores the pool aimlessly. However, by trial 12 (on day 3) if learning has occurred, the rat will navigate directly to the platform. If learning impairments exist, the rat will take a less direct route to the platform.
Figure 1.3 Synaptic versus extrasynaptic NMDA receptor activation of ERK1/2 (modified from Hardingham, 2006). Synaptic NMDA receptors couple strongly to Ras activation, with Ca\(^{2+}\)-dependent activation of Ras-GRF being a prime mediator of this (although other mechanisms are also likely to contribute, such as activation of proline-rich tyrosine kinase 2 (PYK2) by Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII)). In contrast, extrasynaptic NMDA receptors cause inactivation of Ras and ERK. While the molecular basis of this is unclear, several Ca\(^{2+}\)-dependent, NMDA receptor-associated signaling molecules are known to inhibit Ras and ERK. The ERK-inactivating striatal-enriched tyrosine phosphatase (STEP) is activated by the Ca\(^{2+}\)-dependent phosphatase calcineurin, while the Ras-inactivating synGAP is *activated* by CaMKII. Differences between the synaptic and extrasynaptic NMDA receptor signaling complex may explain their opposing influence on the Ras–ERK signaling pathway.
NLRPs are the chief platforms of the inflammasomes. Their amino-terminal sequence is characterized by the presence of a pyrin domain (PYD), followed by a NACHT domain, a NACHT-associated domain (NAD) and several leucine-rich repeats (LRRs). The NACHT domain is responsible for the oligomerization of the inflammasomes. NALP1 contains an additional FIIND domain of as yet unknown function, and a caspase-recruitment domain (CARD), both of which are absent from the other NLRPs.

Panel B (modified from Tschopp et al., 2003) Recognition of lipopolysaccharide (LPS) by Toll-like receptor (TLR)-4 is aided by two accessory proteins known as CD14 and MD-2. TLR-4 contains extracellular leucine-rich repeats (LRRs) and an intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain, and transmits signals through the two TIR adaptor proteins MYDD8 and TIRAP (Toll/IL-1 receptor domain-containing adaptor protein). This leads to the activation of the p38, c-Jun N-terminal kinase (JNK) and nuclear factor B (NF-B) signaling pathways. Caspase-1 is activated by an unknown mechanism, then active caspase-1 cleaves the inactive precursor of IL-1 (pro-IL-1) downstream from the aspartate residue 116 within the substrate-recognition sequence YVAD, thereby liberating the active form of IL-1. Processing of IL-1 is associated with its release into the extracellular space, where it initiates the inflammatory response. In certain situations, active caspase-1 can also trigger apoptosis through cleavage and activation of the downstream caspase-3.
Chapter II

AGE-RELATED INCREASE IN INFLAMMASOME ACTIVITY

Summary

Members of the mammalian nucleotide binding domain, leucine-rich repeat (LRR)-containing receptor (NLR) family of proteins are key modulators of innate immunity regulating inflammation. Our previous work has shown that among the members of this family, NLRP1/NALP1, present in neurons, plays a crucial role in inflammasome formation and the production of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 after acute central nervous system (CNS) injury. Here, we investigated whether age-related cognitive decline may involve a heightened inflammatory response associated with activation of the NLRP1 inflammasome in the hippocampus. Young (3 month) and aged (18 month) male Fischer 344 rats were tested in a spatial acquisition task via Morris water maze. Following behavioral testing, hippocampal lysates were assayed for expression of NLRP1 inflammasome components and inflammatory cytokines. Hippocampal lysates from aged rats showed significantly higher levels of NLRP1 inflammasome constituents, caspase-1, caspase-11, the purinergic receptor P2X7, pannexin-1 and XIAP than lysates from younger animals. Consistent with increases in IL-1β and IL-18 that have been previously shown to correlate with spatial learning deficits. Probenecid, an inhibitor of inflammasome activation, was given at a dose of 0.5 mg/kg twice daily. Treatment began one day before behavioral testing and lasted 3 days. Probenecid treatment reduced activated caspase-1 and ameliorated spatial learning deficits in aged rats. Thus, aging processes stimulate activation of the NLRP1 inflammasome and secretion of IL-1β and IL-18 that may
contribute to age-related cognitive decline in the growing elderly population. Moreover, Probenecid may be potentially used as a therapy to improve cognitive outcomes in the aging population.

**Background**

Inflammatory immune responses are induced in the immune privileged central nervous system (CNS) by aging and neurodegenerative diseases, but the CNS cells and molecular mechanisms that regulate innate immunity are poorly defined. One prominent and early inflammatory cytokine present in aged and diseased tissue is interleukin-1β (IL-1β) that may contribute to secondary cell death. Despite the central function attributed to the immune system in aging and neurodegenerative diseases, the initiating signaling pathways that ultimately lead to the activation of the CNS innate immune system are unclear.

The inflammatory cytokine IL-1β is normally produced and stored in the cytosol as inactive pro-IL-β that is rapidly cleaved by caspase-1, which then elicits inflammation after being released from the cell. A number of host-derived molecules that alert the innate immune system to cell injury and tissue damage activate the inflammasome (Taniguchi and Sagara, 2007). These molecules called “danger-associated molecular patterns” include ATP (McDermott and Tschopp, 2007) and high extracellular potassium (Silverman et al., 2009). Our recent work has shown that the NLRP1 inflammasome in neurons plays a crucial role in the innate CNS immune response induced by injury (de Rivero Vaccari et al., 2008, 2009; Albulafia et al., 2009; Silverman et al., 2009). It is not known whether the NLRP1 inflammasome contributes to inflammatory cytokine production and age-related cognitive impairment.
Amplified and prolonged inflammatory responses in the aged brain have been reported to promote cognitive and behavioral impairments (Godbout et al., 2005; Barrientos et al., 2005), whereas clinical studies of elderly patients with infections reveal increases in the occurrence of delirium (Jackson et al., 2004, Wofford et al., 1996). Moreover, elderly patients with prolonged infection are also prone to an increased probability of developing dementia (George et al., 1997) and increased mortality rate (Rockwood et al., 1999). Thus, the prolonged, amplified production of inflammatory molecules may underlie a heightened neuroinflammatory response in the aged brain leading to cognitive impairments.

In the present study, we found that the NLRP1 inflammasome is involved in age-induced activation of caspase-1, and the release of mature IL-1β and IL-18 in the hippocampus. Moreover, aging induced increased expression and altered cellular distribution of critical components of the NLRP1 inflammasome in hippocampal neurons. These changes corresponded to age-related cognitive deficits in spatial learning. Treatment with probenecid, an anti-inflammatory drug commonly used for gout, reduced NLRP1 inflammasome activation and improved spatial learning performance in aged rats. Thus, it appears that heightened NLRP1 inflammasome activity is induced by the natural aging process resulting in increased inflammatory cytokine production and cognitive impairment that can be attenuated by inhibition of inflammasome activation.

**Materials and methods**

**Animals**

Young (3 months, 220-250 grams) and aged (18 months, 375-450 grams) male Fischer rats were acquired from Charles River Laboratories and the National Institute on
Aging Colony respectively. Animals were maintained on a 12:12 h (light: dark) cycle and given food *ad libitum*. All animals were housed according to National Institutes of Health and United States Department of Agriculture guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. For behavioral testing, n=20/age group. For biochemical analyses, n=6/age group.

**Probenecid treatment**

Eighteen month male Fischer 344 rats were randomized to receive probenecid (Alfa Aesar) or saline vehicle at a dose of 0.5mg/kg by intraperitoneal (I.P.) injection twice daily for 3 days.

**Behavioral Testing**

Spatial acquisition and retention was assessed using a Morris water maze navigational task (Morris, 1981). A circular tank (122 cm diameter) in a room with visual cues was filled with water (21°C) made opaque with white paint. A platform (9.3 cm diameter) hidden just beneath the water surface was placed in the northeast quadrant. The path length (i.e., the distance traveled until locating the platform) and latency to find the platform were recorded and analyzed with Ethovision software (Noldus Information Technology). Latency and mean path length to platform was measured for days 1 – 3 of testing. Each day, the animals were given four trials and an average was determined. The animal was released randomly at each of four starting points (north, south, east, and west) and allowed 60 s to find the hidden platform. After locating the platform, the animal remained on the platform for 10 s. If the animal did not locate the platform within 60 s, it was placed on the platform for 10 s. Following each trial, the animal was placed in a cage kept warm with an infrared heating lamp. Inter-trial interval was approximately 4 min.
Testing days 1-3 for latency and day 3 for path length are reported for retention of the task. For probenecid experiment, animals were tested on days 1 – 3 and 8 – 10. Probenecid or saline vehicle was administered on days 7 – 9. Behavioral testing was conducted similarly to the testing above with the placement of the hidden platform moved to the southwest quadrant for the second testing session. Latency to platform is reported for days 1-3 and 8-10. Mean path lengths are reported for day 10.

**Immunoblotting**

Animals were anesthetized with 3% isoflurane/70% N₂O/30% O₂ for 5 minutes then sacrificed by decapitation. The bilateral hippocampi were dissected out at 4°C in saline and frozen in liquid nitrogen within 3 min of decapitation, and stored at -80 deg C. For detection of inflammasome components, receptors and inflammatory cytokines, a section of the right hippocampus of young and aged animals was homogenized in extraction buffer (20mM Tris–HCl, pH: 7.5, 150mM NaCl, 1% Triton X-100; 1mM ethylenediaminetetraacetic acid, 1mM ethylene glycol tetraacetic acid, 2.5mM pyrophosphate, 1mM -glycerophosphate) with protease and phosphatase inhibitors cocktail (Sigma). Proteins were resolved in 10–20% Tris–HCl Criterion precasted gels (Bio-Rad), transferred to polyvinylidene difluoride membranes (Applied Biosystems) and placed in blocking buffer (PBS, 0.1% Tween-20, 0.4% I-Block (Applied Biosystems) and then incubated for 1 h with: anti-IL-1β (1:1000, National Cancer Institute - 3ZD MAb), anti-IL-18 (1:1000, Abcam ab37640), anti-caspase-1 (1:1000, Imgenex - IMG5028), anti-caspase-11 (1:1000, Alexis Biochemicals, Axxora - ALX-210-818), anti-ASC (1:5000, Bethyl Laboratories, as described in de Rivero Vaccari et. al. 2007), anti-NLRP1 (1:1000, Bethyl Laboratories as described in de Rivero Vaccari *et al.*, 2007), anti-pannexin-1
(1:1000, Invitrogen - 488100) and anti-P2X7 (1:1000, Alomone Labs - APR-004) followed by appropriate secondary horseradish peroxidase (HRP)-linked antibodies (Cell Signaling). Visualization of signal was enhanced by chemiluminescence using a phototope-HRP detection kit (Cell Signaling). To control for protein loading, immunoblots were stripped with Restore, Western blot stripping buffer (Pierce) and blotted for β-tubulin (1:5000, BD Biosciences Pharmingen). Quantification of band density was performed using UN-Scan-IT gel™ quantifying software (Silk Scientific), and data were normalized to β-tubulin. However IL-1β and IL-18 immunoblots were not quantified because these two proteins are secreted cytokines.

**Perfusion Fixation**

Following behavior testing, a separate group of young and aged animals were anesthetized with 3% isoflurane/70% N₂O/30% O₂ for 5 minutes and intracardially perfused with cold heparinized normal saline solution and subsequently with 4% paraformaldehyde solution. Brains were removed and placed in 4% paraformaldehyde at 4°C for 20 h. Next brains were placed in 20% sucrose in 0.1M PBS and stored at 4°C until sectioning through the hippocampus from 2.4 to 5.8 mm posterior to bregma. Sections were taken with a thickness of 50 µm.

**Immunohistochemistry**

Frozen sections were blocked by treatment with normal goat serum (Vector Laboratories), rinsed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) and incubated overnight at 4°C with primary antibodies against the same proteins analyzed by immunoblotting at a dilution of 1:500. To determine the precise cellular distribution of caspase-1, pannexin-1 and P2X7, sections were double stained with the neuron specific
marker mouse anti-neuronal nuclei (NeuN; Chemicon) and anti-caspase-1 (Upstate), anti-P2X7 (Alomone Labs) and anti-pannexin-1 (Invitrogen). Alexa-Fluor secondary antibody conjugates (Molecular Probes) were used as secondary antibodies. Controls using an irrelevant antibody of the same isotype were run in parallel to evaluate antibody specificity.

Statistical Analysis

Data are expressed as mean +/- standard error mean (SEM). Statistical comparisons between young and aged groups, and treatment groups were made using one-way analysis of variance (ANOVA), or repeated measures analysis of variance followed by Tukey's multiple comparison tests depending on the outcome measure. P-values of significance used were *p < 0.05.

Results

Aged animals perform poorly on a hippocampal-dependent spatial learning task

To assess age-related deficits in hippocampal-dependent spatial learning, young and aged rats were tested in a 3-day spatial acquisition task via Morris water maze (Fig. 1). Mean (+/- S.E.M.) values were determined for each testing day on latency to platform (Fig.1A) and for testing day 3 for mean path length (Fig.1B) For days 2 and 3, young rats located the hidden platform with lower latencies (day 2: 17.1 +/- 1.4 s, day 3: 9.4 +/- 0.7 s) than aged rats (day 2: 35.8 +/- 1.3 s, day 3: 29.3 +/- 1.4 s) (Fig. 1A). Additionally, mean path-length for young rats (502 +/- 48 cm) was significantly shorter than for aged rats (1487 +/- 132 cm) on day 3 of testing. No differences were detected in swim speeds between groups (data not shown). Aged rats are consistently impaired in learning the location of the escape platform (reviewed in Rosenzweig and Barnes, 2003). Therefore,
our results were similar to previous findings that aged rats demonstrate spatial learning impairment compared to young rats.

**Aging induces increases in active IL-1β and IL-18 in the hippocampus**

Previous findings have shown that IL-1 cytokines in the brain are associated with the aging process. To establish whether aging activates these pro-inflammatory cytokines in the hippocampus, protein lysates from young and aged rats were analyzed for IL-1β and IL-18 by immunoblotting procedures. As shown in Fig. 2, the levels of active, processed forms of IL-1β and IL-18 were higher in the aged animals than their younger counterparts, thus indicating that aging induces activation of these inflammatory cytokines in the hippocampus. Unlike IL-1α, IL-1β and IL-18 are cytokines that are active only after inflammasome processing. Therefore, since IL-1β and IL-18 are secreted cytokines, quantification of these cytokines was not conducted for it would represent an inaccurate estimation due to the inability to determine the amount of IL-1 cytokines that are still in the cell and the amount that has been secreted. Instead, inflammasome activation was determined by measuring the protein levels of caspase-1 (see below).

**Aging increases hippocampal expression of NLRP1 inflammasome components in the hippocampus.**

The NLRP1 inflammasome in neurons constitutes a multiprotein complex consisting of caspase-1, caspase-11, ASC, NLRP1, P2X7, pannexin-1 and XIAP (de Rivero Vaccari *et al.*, 2008). Upon inflammasome activation caspase-1 is cleaved into active fragments that process pro-IL-1β and pro-IL-18 into active IL-1β and IL-18 (Dinarello, 2009). To determine whether aging alters expression of NLRP1
inflammasome components, protein lysates of young and aged hippocampi were analyzed for levels of inflammatory caspases and key inflammasome proteins (Fig. 3). Hippocampal lysates from aged animals contained significantly higher levels of cleaved fragments of caspase-1 (p=0.006, Fig. 3A), caspase-11 (p=0.011, Fig. 3B), and XIAP (p=0.0021, Fig 3G), and increased levels of the P2X7 receptor (p=0.001, Fig. E) and pannexin-1 (p=0.049, Fig. 3F) than lysates from young rats. Levels of NLRP1 (Fig. 3C) and ASC (Fig. 3D) were not altered in the aged animals. Thus, aging stimulates the expression of some of the key components of the NLRP1 inflammasome in the hippocampus, suggesting an involvement of the inflammasome in the aging process.

**Aging induces alterations in expression patterns of pannexin-1 and the purinergic receptor P2X7 in the hippocampus.**

In previous work, it was reported that the pore-forming protein pannexin-1 can transport extracellular NLRP1 stimuli into the cytoplasm of neurons and astrocytes after P2X7 receptors bind ATP (Silverman et al., 2009), suggesting that K⁺ ion influx through pannexin-1 pores may initiate activation of the NLRP1 inflammasome. To establish whether aging induces changes in the pattern of expression of caspase-1, pannexin-1 and the P2X7 receptor, hippocampi from young and aged rats were examined by confocal microscopy. Brain sections from aged rats underwent immunohistochemical procedures followed by confocal microscopy. Images indicate that aging results in altered staining patterns of inflammasome proteins in the hippocampus (Fig. 4). In young rats, caspase-1 immunoreactivity (red) was present in NeuN positive nuclei (green) in a diffuse expression pattern, while intense caspase-1 staining was seen in hippocampal neurons of aged rats located in the cell nucleus and cytoplasm. A more striking alteration was
observed in the immunostaining of pannexin-1 and P2X7 receptors. In aged rats, immunoreactivity of both inflammasome proteins was markedly enhanced, and intense patchy staining was seen in the neuronal soma near or associated with the plasma membrane (arrows). These patches exhibited a polarized distribution in the hippocampal neuronal membrane (arrows). These results demonstrate that aging induces dramatic alterations in the expression patterns of caspase-1, pannexin-1 and P2X7 receptors in hippocampal neurons.

**Probenecid treatment attenuates age-related elevation in NLRP1 inflammasome components**

Probenecid was previously shown to block inflammasome activity, specifically by inhibiting Pannexin 1 (Silverman *et al.*, 2008). Here we show a significant decrease in cleaved caspase-1 (p=0.0426, Fig.5B), P2X7 receptor (p=0.0258, Fig.5C), and Pannexin1 (p=0.004, Fig.5D) protein expression in the hippocampus following probenecid treatment in aged rats consistent with lower protein levels of IL-1β and IL-18. Treatment with probenecid also improved spatial learning performance in aged rats. Probenecid treated aged rats demonstrated lower latency to platform (p=0.0287, Fig.5E) and mean path length (p=0.015, Fig. 5F) than vehicle-treated age-matched controls. These results demonstrate that treatment with probenecid reduces age-related increases in NLRP1 inflammasome activation in the aged hippocampus and ameliorates age-related cognitive deficits.
Discussion

In this study we have shown for the first time an age-related heightened activation of the NLRP1 inflammasome system associated with increased inflammation and cognitive impairment in the hippocampus of aged rats. Our data show that aging promotes NLRP1 inflammasome activity resulting in processing of caspase-1 and upregulation of caspase-11 leading to maturation of IL-1β and IL-18. The neuronal NLRP1 inflammasome is a multiprotein complex consisting of inflammatory caspases-1 and -11, NLRP1, the adaptor protein ASC and the inhibitor of apoptosis protein XIAP. In addition, we have previously reported that the NLRP1 inflammasome interacts with the pore forming protein pannexin-1 and the purinergic receptor P2X7 (de Rivero Vaccari et al., 2008, 2009; Silverman et al., 2009). Aging leads to significant cognitive impairment in hippocampal dependent spatial learning tasks (reviewed in Rosenzweig and Barnes, 2003) that may be associated with increased inflammatory cytokine production resulting from NLRP1 activation. Thus, the NLRP1 inflammasome constitutes an important arm of the innate CNS inflammatory response associated with aging in the hippocampus.

IL-1β is synthesized by neuronal and glial cells (Lechan et al., 1990) and is released in response to injury, insult and stress (Benveniste, 1992; Bhat et al., 1996; Dinarello, 2004, 2006). We found that hippocampal neurons, which are generally not associated with immune functions, express NLRP1 inflammasome proteins. These results are in agreement with previous findings that show NLRP1 expression in neurons of the spinal cord (de Rivero Vaccari et al., 2008) and cerebral cortex (de Rivero Vaccari et al., 2009; Abulafia et al., 2009) after injury. Moreover, the cellular distribution of NLRP1 inflammasome proteins changed during the aging process. Our results suggest
that NLRP1 inflammasome activity is fundamental for the processing of IL-1β and IL-18 and for the innate inflammatory response in aged neurons. However, ASC, caspase-1 and caspase-11 are also present in astrocytes, oligodendrocytes and microglial cells. Therefore, characterization of the composition and subcellular localization of inflammasomes in glia may provide a clearer insight into the mechanisms leading to cytokine secretion and cell death caused by caspase-1 with aging. For example, aging-induced NLRP1 inflammasome activation in neurons could lead to reactive gliosis in neighboring cells mediated by IL-1 cytokines released from neurons.

The inflammasome in hippocampal neurons is a protein complex containing NLRP1 as a scaffolding protein that activates caspase-1 to promote IL-1β maturation associated with aging. Although the total levels of NLRP1 and ASC in lysates did not change significantly with aging, the proportions of other key components that form the NLRP1 inflammasome increased. These findings are in agreement with our previous work on NLRP1 inflammasome regulation following SCI (de Rivero Vaccari et al., 2008) and TBI (de Rivero Vaccari et al., 2009) that showed similar changes in NLRP1 components after injury. Moreover, the inflammasome in normal tissues is speculated to be in an inactive state by binding to a putative inhibitor (Dinarello, 2005), but the nature of this inhibitor has not been identified. We previously suggested that XIAP in the NLRP1 inflammasome complex may inhibit caspase-1 activity preventing the activation and processing of IL-1β and IL-18 (de Rivero Vaccari et al., 2008, 2009; Silverman et al., 2009). Moreover, aging-induced activation of the inflammasome was found to be associated with cleavage of XIAP into fragments. Cleavage of XIAP produces an N-terminal BIR1-2 fragment with reduced ability to inhibit caspases (Katz et al., 2001;
Keane et al., 2001; Lotocki and Keane, 2002). Therefore, aging-related XIAP cleavage may reduce the threshold for activation of caspase-1, leading to processing and secretion of IL-1β and IL-18.

Our results are in agreement with earlier studies that demonstrate systemic administration of IL-1β results in impaired hippocampal-dependent consolidation of memories in a fear-conditioning paradigm (Pugh et al., 1999; Thomson and Sutherland, 2005; Barrientos et al., 2002). Neutralization of IL-1β blocked the deficits in hippocampal-dependent memory consolidation (Pugh et al., 1999; Gonzalez et al., 2009). Other reports demonstrate that the aging process results in elevated concentrations of IL-1β at 15 months of age (Griffin et al., 2006), whereas hippocampal IL-18, a closely related IL-1 proinflammatory cytokine increases in rats as early as 9 months of age (Griffin et al., 2006). Additionally, the age-specific elevation in these proinflammatory cytokines may influence deficits in long-term potentiation (Lynch and Lynch 2002; Maher et al., 2005). Further studies are required to determine whether the NLRP1 inflammasome-induced activation and increased IL-1β and IL-18 observed in our study influence deficits in synaptic transmission leading to cognitive decline.

To date, microbial pathogen-associated molecules and toxins have been discussed as key triggers of activation of inflammasomes (Taniguchi et al., 2007; McDermott et al., 2007). However, recently, environmental (Faustin and Reed, 2008; Dostert et al., 2008; Li et al., 2008) and neurodegenerative (Salminen et al., 2008; Halle et al., 2008) stimuli have been identified that lead to IL-1β release by means of inflammasomes. With respect to the latter, Halle et al. (2008) demonstrated that the NLRP3 inflammasome is activated by fibrous particles of amyloid-beta that results in cleavage of caspase-1 and production
of IL-1β in microglia and macrophages. However, it is not known whether amyloid-beta activates the NLRP1 inflammasome in neurons thus further enhancing production of IL-1β in the aging brain. Our recent work demonstrated that the pore-forming protein pannexin-1 in neurons and astrocytes transports the extracellular K⁺ ions to stimulate the NLRP1 inflammasome in the cytoplasm after P2X7 receptors bind ATP (Silverman et al., 2009). Thus, it is possible that the aging-induced increases in pannexin-1 and P2X7 expression in hippocampal neurons observed in the present study may facilitate K⁺ influx, thereby initiating NLRP1 inflammasome activation.

Probenecid, regularly used in the treatment of gout, was previously identified as an inhibitor of inflammasome activation in neurons and astrocytes (Silverman et al., 2008; 2009). Our findings support the use of probenecid as an anti-inflammatory therapy in the CNS. The aging-induced elevation in inflammasome activation in the hippocampus was attenuated by probenecid treatment, resulting in improved cognitive outcomes in vivo. Our results suggest that inhibition of inflammasome activation in the aged brain reduces caspase-1 activation and, in turn, reduces inflammatory cytokines, IL-1β and IL-18, which have been linked to age-related cognitive decline. Therapeutic interventions that inhibit the NLRP1 inflammasome, with corresponding reduction in proinflammatory cytokines, may improve overall cognitive outcomes and reduce symptoms of normal cognitive aging.

IL-1β and IL-18 contribute to the pathology of several neurodegenerative diseases such as Alzheimer disease (Di Bona et al., 2008; Chiarini et al., 2006) and Parkinson disease (Tansey et al., 2007). Since production of these cytokines is involved in a positive feedback mechanism in which more cytokines are produced upon cytokine
release (Vannier and Dinarello, 1994), it is reasonable to hypothesize that interfering with inflammasome activation may prove to be beneficial in delaying development of age-related neurodegenerative diseases in which the IL-1 inflammatory response plays a pathogenic role.
Figures for Chapter II

Figure 2.1 Aged animals perform poorly on hippocampal dependent spatial learning task. (A) Average latency to platform on day 1-3 of spatial acquisition testing via Morris water maze from young (open circles) and aged rats (closed circles). (B) Mean path length for young and aged rats on final day of testing. [Data are presented as mean +/- SEM *P<0.01 compared to young. n = 20 per group.]

Figure 2.2 Aging induces processing of IL-1β and IL-18 in the hippocampus. Representative immunoblot analysis of hippocampal brain lysates of young (Y) and aged (A) animals. Brain lysates were immunoblotted with antibodies against IL-1β and IL-18. β-Tubulin was used as internal standard and control for protein loading. [n=6 per group.]
Figure 2.3 Aging alters expression of NLRP1 inflammasome components. Representative immunoblot analysis of (A) caspase-1, (B) caspase-11 (C) NLRP1, (D) ASC, (E) cleaved XIAP, (F) P2X7 receptor, and (G) pannexin-1 in brain lysates of young (Y) and aged (A) animals. Protein levels of cleaved caspase-1 and -11 are higher in aged animals compared to young. Protein levels of NLRP1 and ASC did not change with age whereas P2X7 receptor, the pannexin-1 protein and the cleaved fragment of XIAP are higher in aged animals than in young animals. β-Tubulin was used as internal standard and control for protein loading. [Data are presented as mean +/- SEM *p<0.05, **p<0.01, ***p<0.005 compared to young. n=6 per group.]
Figure 2.4 Caspase-1, pannexin-1 and P2X7R are present in hippocampal neurons and aging induces alterations in the patterns of protein expression.
Confocal images show hippocampal neurons in the CA3 region of young and aged animals. Sections were stained for caspase-1, pannexin-1 and P2X7 (red) and the neuronal marker NeuN (green). In aged animals, the immunoreactivity of caspase-1, P2X7 and pannexin-1 are increased in neurons in the hippocampus compared to young animals with P2X7 and pannexin-1 showing a polarized distribution near the cell membrane (arrows). Bar = 20µm.
Figure 2.5 Probenecid reduces protein levels of key NLRP1 inflammasome components in the aged rats hippocampus and ameliorates age-related spatial learning deficits.

(A-D) Immunoblot analysis of cleaved caspase-1, pannexin1 and P2X7R in hippocampal lysates of vehicle (Veh)-treated and probenecid (Pr)-treated 18-month-old rats. β-tubulin was used as an internal control. (A) Representative immunoblots of inflammasome related proteins. (B) Densitometric analysis of immunoblots from brain lysates of cleaved caspase-1, (C) P2X7R, and (D) pannexin1. (E-F) Young and aged animals underwent behavioral testing following either probenecid or vehicle treatment. (E) In a hippocampal-dependent spatial learning task via Morris water maze latency to platform was measured on days 1-3 and 8-10. Probenecid-treatment improved latency to platform measured on the final day of testing (F) Mean path length was determined on day 10 of testing and probenecid-treated rats demonstrated significantly shorter mean path lengths than vehicle-treated controls. Drug treatment was administered twice daily for 3 days (days 7-9). Data are presented as mean +/- SEM *p < 0.05, **p < 0.005 compared to vehicle. N = 6-8/per group.
Chapter III

ISO-INDUCED CELL DEATH INVOLVING NR2B-NMDAR

Summary

Isoflurane anesthesia exhibits inherent neurotoxicity to cultured cells. Caspase-3 activation has been implicated in isoflurane-induced apoptotic cell death. However, the underlying mechanism remains unclear. Here, we treated cultured neurons (E15 from mice cortex and hippocampus) with 1% isoflurane. Immunoblotting revealed increases in caspase-3 activation and protein expression of NMDA receptor subunits NR2B and NR1. Cell death assays demonstrated isoflurane-induced cell death, which was attenuated by treatment with Ro 25-6981 (Ro), an NR2B specific NMDA receptor antagonist. Taken together, our findings suggest that isoflurane-induced cell death involving caspase-3 activation may be mediated by NR2B-containing NMDA receptors.

Background

Isoflurane, a common inhalation anesthetic, has been linked to increased levels of inflammation (Wu et al., 2010), cell death via apoptosis (Zhang et al., 2008; 2010) and promotion of Alzheimer’s disease pathology (Bianchi et al., 2010; Perucho et al., 2010; Zhen et al., 2009). Isoflurane is also implicated in neurotoxicity in the developing brain. The underlying mechanisms of isoflurane-induced cell death are largely unknown. The array of potential neurotoxic mechanisms of isoflurane may be a consequence of the various neurotransmitter systems modulated by the anesthetic. The mechanism of anesthetic action for isoflurane involves inhibition of synaptic neurotransmission by potentiating γ-amino-butyric-acid type A (GABAA) and inhibition of glutamate NMDA receptors (Hoffman et al., 1992; Franks and Lieb, 1994). Inhibition of NMDA receptors...
may have deleterious effects on neurons, leading to disruption of NMDAR-mediated signaling pathways and potentially cell death processes. Over-stimulation of the NMDA receptor mediated by caspase-3 activation has been previously shown to lead to cell death (Laabich and Cooper, 2000; Laabich et al., 2000; 2001; Lin et al., 2005). Caspase-3 activation has been implicated in isoflurane-induced cell death in organotypic hippocampal slice cultures (Sanders et al., 2009), primary neurons (Head et al., 2009), postnatal day (PND) 5-7 mice (Head et al., 2009) and PND7 rats (Jevtovic-Todorovic et al., 2003). It is possible that the blockade of NMDA receptors by isoflurane may induce caspase-3 mediated cell death. Here, we show caspase-3 activation in isoflurane-treated neurons in culture. Additionally, Ro 25-6981, an NMDA receptor antagonist specific for the NR2B subunit, reduces isoflurane-induced cell death. Thus, NR2B-mediated isoflurane-induced cell death involving caspase-3 activation may contribute to the damaging effects of isoflurane in vitro.

Materials and methods

Animals

Procedures involving animals were conducted in conformity with National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Neuronal cultures were prepared from embryonic day 16-17 mice cortices and hippocampi as previously described (Keane et al., 1992; Tedeshi et al., 1986). Cortical and hippocampal tissue was disrupted into a cell suspension by gentle titration and seeded in 60-mm dishes at a density of 2 x 10^6 cells/dish. Neurons were grown on poly-L-lysine-coated tissue culture dishes in N5 medium that contained 5% serum fraction (Kawamoto and Barrett, 1986). Neurons were maintained for 14 days.
Cell death assay

The percentage of dead cells was evaluated using a multi-well fluorescence plate reader. Hippocampal neurons were cultured in a 96-well plate for 14 days. Cells were treated with either Ro 25-6981 (Ro, 1M) Isoflurane (Iso, 1%), Ro + Iso, or no treatment (control). Propidium iodide (PI, 15µm, Sigma, St. Louis, MO, USA) was added to cells to label nuclei of dead cells. The fluorescence intensity from the dead cells was measured using 530-nm excitation and 645-nm emission filters. Readings were taken at 30min, 1h, 2h, and 3h after initial treatment. Cultures were stored at 37°C between measurements. DMSO (dimethyl sulfoxide) was added to each well to permeabilize all cells and label all nuclei with PI. At 10m and 24h after DMSO, fluorescent intensity was re-measured to obtain a value corresponding to total cells. The percentage of dead cells was calculated as the proportion of fluorescence intensity of dead cells to that of total cells.

Isoflurane treatment

For isoflurane treatment, the medium of cell cultures was removed and replaced with medium containing 1% isoflurane for 30 minutes, 1h or 2h, whereas controls received medium alone. Cells were washed once in ice-cold PBS and lysed as previously described (Keane et al., 1997) and prepared for immunoblot analysis.

Antibodies and Immunoblotting

Mouse monoclonal antibodies against NR2B (1:1000, Cell Signaling Technologies), and β-actin (1:5000, Sigma), polyclonal antibodies against NR2A (1:1000, Cell Signaling Technologies), NR1 (1:1000, Cell Signaling Technologies) and Caspase-3 (1:1000 Cell Signaling Technologies) were used. For immunoblotting, primary cell cultures were lysed in lysis buffer (20mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA,
1mM EGTA, 1% Triton X-100, 2.5mM pyrophosphate, 1mM β-glycerophosphate) with protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor cocktail 3 (Sigma-Aldrich). Cells were spun at 12,000 x g for 3 min, and samples were taken from the supernatant, avoiding both the pellet and the lipids on the surface. Laemmli sample buffer was added. Proteins were resolved in 10–20%, or 10% Tris-HCl Criterion pre-casted gels (Bio-Rad, Hercules, CA), transferred to polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA), placed in blocking buffer (PBS, 0.1% Tween 20, and 0.4% I-Block (Applied Biosystems)), then membranes were incubated for 1h with primary antibodies followed by appropriate HRP-linked antibodies (Cell Signaling Technology). Visualization of signal was enhanced by chemiluminescence using a phototope-HRP detection kit (Cell Signaling Technology). To control for protein loading, immunoblots were stripped with Restore Western blot stripping buffer (Pierce, Rockford, IL), and blotted for β-actin using monoclonal anti-β-actin antibody (1:5000; Sigma). Quantification of band density was performed using the NIH ImageJ 1.34 software, and data were normalized to β-actin.

**Statistical analysis**

Cultures were randomized to receive 1% isoflurane/media mixture for 30 minutes, 1h or 2h, or medium only. Data are expressed as mean +/-SEM. Statistical comparisons between experimental and control groups were made using repeated measures ANOVA with Tukey’s multiple comparisons post-hoc analysis, or repeated measures analysis of variance depending on outcome measure. P-values for significance used were P<0.05.
Results

Protein expression of caspase-3 fragment and NMDA receptor subunits NR1 and NR2B

In Figure 3.1, immunoblotting showed that isoflurane exposure increased levels of caspase-3 fragment (~17kD) in hippocampal and cortical neuronal cultures. We assessed whether isoflurane induced caspase-3 activation, a final step in the cascade of apoptosis (Thornberry, 1998). Isoflurane exposure of cortical neurons for 30min and 1h induced increases in caspase-3 cleavage (p=<0.001, p=0.004 respectively, Fig.3.1B). Caspase-3 activation returned to baseline with 2h of isoflurane exposure in cortical cultures. In hippocampal cultures, 2h of isoflurane exposure induced caspase-3 cleavage compared to controls (p=0.0043, Fig. 3.1B). Quantification of the Western blot of NR2B (Fig. 3.1C) revealed a significant increases in NR2B in cortical cultures exposed to isoflurane for 30m (p=0.0046), 1h (p=0.0037), and 2h (p=<0.001), and in hippocampal cultures exposed to isoflurane for 2h (p=<0.001). Quantification of immunoblots of NR1 (Fig. 3.1D) revealed a significant increase in protein expression levels in cortical and hippocampal cultures exposed to isoflurane for 2 hours (p=0.004, p=0.003 respectively).

Cell death assays for isoflurane, Ro 25-6981, and Ro + Iso in neuronal cultures

Cell death was assessed using propidium iodide (PI) and multi-well fluorescence readings in Figure 3.2. Wells were treated with Ro, Iso, Ro + Iso or no treatment (control). Fluorescence readings were taken at 30m, 1h, 2h, and 4h post-treatment. DMSO was added after 4h reading to permeabilize all cells, and PI labeled all nuclei to determine total cells. A percentage of dead cells to total cells was determined and plotted for each time point. Isoflurane induced significant cell death compared to control (p=0.025). Ro
treatment significantly reduced isoflurane-induced cell death \((p=0.008)\) in neuronal cultures. Interestingly, Ro treatment also reduced cell death compared to control \((p=0.006)\).

**Discussion**

Caspase-3 plays a central role in apoptosis, cleaving and activating caspases 6, 7, and 9, while caspase-3 itself is cleaved by caspases 8, 9, and 10. The role of caspase-3 in the caspase apoptotic pathway involves amplification at a late stage leading to cell death (Slee *et al.*, 1999). We were specifically interested in determining the involvement of NR2B-containing NMDA receptors in caspase-3 mediated isoflurane-induced cell death. An increase in NMDA receptor subunits NR2B and NR1 was detected in hippocampal and cortical cultures treated with the anesthetic. Our findings are supported by previous reports of an isoflurane-induced upregulation of NR2B in mice (Rammes *et al.*, 2009). However, in this study, mice experienced improvement in spatial learning and synaptic plasticity. The discrepancy may be a result of differences in methodology, such as age of animals, isoflurane dose and administration, and inherent differences in studies performed *in vivo* and *in vitro*.

Additionally, our results indicate that caspase-3 activation is induced in isoflurane treated neuronal cultures, exhibiting the inherent neurotoxicity of isoflurane anesthesia *in vitro*. Isoflurane-induced caspase-3 activation has previously been shown via an NMDA-mediated apoptotic pathway in mixed neuronal/glial cortical cell cultures (Wise-Faberowski *et al.*, 2006) and can be attenuated by Memantine, an NMDA receptor antagonist (Zhang *et al.*, 2008). Since, isoflurane is a competitive NMDA receptor antagonist (Hoffman *et al.*, 1992), it is possible that blockade of NMDA receptors by the
anesthetic induces caspase-3 mediated cell death. However, we demonstrated a reduction in cell death by treatment with Ro 25-6981, an NR2B specific NMDA receptor antagonist. NMDA-gated currents mediated by NR2B-containing receptors exhibit greater sensitivity to isoflurane than those mediated by NR2A-containing receptors (Ming et al., 2002). The neuroprotective effects of Ro suggest NR2B specificity for isoflurane-induced caspase-3 activation and subsequent cell death.

Our findings suggest that isoflurane-induced cell death induces caspase-3 activation and may be mediated by NR2B-containing NMDA receptors. Moreover, the observed increase in NMDA receptor subunits may further contribute to the activation of the caspase pathway *in vitro*. However, this may not represent an exclusive mechanism of cell damage in anesthesia treatment. The damage to cells by isoflurane may involve various mechanisms of cellular injury, including, but not limited to caspase activation leading to cell death, and increases in NMDA receptor subunits. Isoflurane-induced increases in NMDA receptor subunits may also contribute to a disruption in downstream cell signaling pathways. Future studies are necessary to parse out the contribution of NR2B-containing NMDA receptors and caspase activation in the neurotoxic effects of isoflurane.
Figure 3.1: Immunoblots of cortical and hippocampal neurons treated with isoflurane in vitro. Primary neuron cultures from cortex and hippocampus were treated with isoflurane for 30min, 1h, or 2h, or no isoflurane (control). (A) Representative immunoblots for antibodies against NR2B, NR1, caspase-3, and β-actin are shown. Quantification of immunoblots were performed for (B) NR2B, (C) NR1, (D) cleaved caspase-3. β-actin was used as an internal standard and control for protein loading. Data are presented as means, error bars represent standard error mean (s.e.m.), n=5/group. One-way ANOVA and Tukey’s post-hoc analysis were performed on hippocampal and cortical cultures separately, *p<0.005, **p<0.001.
Figure 3.2: Cell death assays for isoflurane, Ro 25-6981, and Ro + Iso in neuronal cultures.

Primary neuron cultures were treated with isoflurane (1%, Iso), Ro 25-6981 (1mM, Ro), both Ro and Iso, or no treatment (control). Propidium iodide was added to wells, labeling nuclei of dead cells. Fluorescent measurements were taken 30m, 60m, 90m, and 120m after treatment. The percentage of dead cells was calculated from total cells, which were determined by addition of DMSO after 120m. [Data are presented as means, error bars represent standard error mean (s.e.m.), n=11-13/group. Repeated measures ANOVA and Tukey’s post-hoc analysis were performed; *p<0.05, **p<0.01]
Chapter IV

ANESTHESIA-INDUCED INCREASE IN NR2B IN AGED RATS

Summary

Postoperative cognitive dysfunction (POCD) afflicts a large number of elderly surgical patients following surgery with general anesthesia. Underlying mechanisms of POCD remain unclear. NMDA receptors, critical in learning and memory, display protein expression changes with age, while inhalation anesthetics modulate these receptors and consequent signaling pathways. The aim of this study was to identify protein expression changes in NMDA receptor subunits and downstream signaling pathways in aged rats that demonstrated anesthesia-induced spatial learning impairments. Aged (18-month-old) Fischer 344 rats were randomly assigned to receive isoflurane/nitrous oxide anesthesia for 4h or no anesthesia. Spatial learning was assessed at 2 weeks and 3 months post-anesthesia via a 3-day Morris water maze spatial acquisition task. Hippocampal and cortical protein lysates were immunoblotted for caspase 3, NMDA receptor subunits, and phospho-ERK1/2. In a separate experiment, Ro 25-6981 (0.5mg/kg) was administered by I.P. injection 15 minutes before anesthesia to aged rats. Immunoblotting revealed an anesthesia-induced increase in NR2B protein expression corresponding to spatial learning impairment in aged rats 3 months post-anesthesia. Additionally, a reduction in phospho-ERK1/2 in the hippocampus and cortex was detected in these animals. Ro 25-6981 pretreatment attenuated the increase in acute NR2B protein expression. Our findings suggest a role for dysregulation of NMDA receptor mediated signaling pathways in the hippocampus and cortex of aged rats treated with isoflurane anesthesia, leading to
chronic spatial learning deficits. The data also indicate a novel therapeutic intervention for anesthesia associated cognitive deficits.

**Background**

The aged brain is more vulnerable to anesthetic insult when compared to the brain of younger patients, resulting in lasting cognitive impairments for elderly surgical patients. In the clinical population, POCD, observed in 10-25% of elderly patients, involves the manifestation of symptoms weeks to months after the anesthetic agent has cleared the body (Fong *et al.*., 2006; Newman *et al.*., 2007). In a rodent model, isoflurane, a commonly used inhalation anesthetic, induced spatial learning deficits up to 2 months after anesthetic exposure (Culley *et al.*., 2004). A biochemical mechanism for the interaction of anesthesia and the aged brain resulting in POCD remains lacking in the literature, despite the prevalence in humans and rodents.

Isoflurane modulates NMDA receptors (Hoffman *et al.*., 1992). Alterations in protein and mRNA expression of NMDA receptor subunits occur in the aged brain (reviewed Magnusson *et al.*., 2010) that may play a role in anesthesia-induced spatial learning deficits in aged rats. Specifically, elevated levels of NR1 and NR2B protein expression in the hippocampus correspond to poor spatial outcomes in aged mice (Zhao *et al.*, 2009). Here we investigated the effect of isoflurane anesthesia on protein expression levels of NMDA receptor subunits and downstream signaling molecules, ERK1/2 in the hippocampus and cortex of aged rats *in vivo*. The aim of the current study was to identify isoflurane-induced disruption in NMDA-mediated signaling associated with cognitive impairment in the aged rat brain.
Materials and methods

Animals

Experimental procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Miami Animal Care and Use Committee. Male Fischer rats (18 months; 420-480 g) were acquired from the National Institute on Aging Colony at Harlan. This age was chosen because Fischer rats have a median life expectancy of 26 months, and they have been shown to demonstrate age-related cognitive impairment (Frick et al., 1995) similar to the clinical population. Animals were kept on a 12:12 h light: dark cycle, with access to food and water ad libitum.

General anesthesia

After a two week acclimation period under standard laboratory conditions, rats were randomly assigned to receive 1.8% isoflurane-70%, Nitrous oxide (N₂O), 30% Oxygen (O₂) for 4h or no anesthesia. Animals were anesthetized with 3% isoflurane, 70% N₂O, and 30% O₂, and then intubated endotracheally and mechanically ventilated (Harvard Apparatus, Holliston, MA, USA) followed by a reduction to 0.5% isoflurane, 70% N₂O, and 30% O₂. A catheter was inserted into the tail artery to monitor blood pressure and blood gases throughout the procedure. Pancuronium bromide (0.5 mg/kg) was administered through the tail artery to facilitate mechanical ventilation. When blood gases reached target readings (pO₂ = 120-180 mmHg, pCO₂ = 35-45 mmHg), and mean arterial blood pressure stabilized (MAP =120-160 mmHg), deep anesthesia (1.8% isoflurane with nitrous oxide/oxygen mixture) was given for 4h. Blood gases, MAP, whole body and brain temperatures were monitored and maintained at physiological...
levels throughout the general anesthetic exposure. Temperatures were maintained at 37°C ± 0.5°C by heating lamps. After 4hrs, isoflurane was discontinued and, depending on the experimental condition, the animal was decapitated or ventilated on 30% O₂ and 70% N₂O until able to respire independently and placed back into its home cage. Naïve rats were kept in their home cage, in the same room as experimental animals for the duration of anesthetic exposure with no anesthesia.

**Spatial learning behavioral testing**

The spatial reference memory version of the Morris water maze (Morris *et al*., 1986) is a standard task used to assess hippocampal-dependent spatial learning in rodents. Spatial acquisition and retention was assessed using several water maze navigational tasks. A circular tank (122 cm diameter) placed in a room with visual cues was filled with water (21°C) made opaque with white paint. A platform (9.3 cm diameter) hidden just beneath the water surface was placed in the northeast quadrant for testing. The path length (i.e., the distance traveled by the rat in the water maze until locating the platform) and latency to find the platform were recorded with an automated tracking system (Ethovision). During baseline testing, the animals were tested over 3 days prior to anesthesia exposure. Each day, the animals were given four trials. The animal was placed randomly at each of four starting points (north, south, east, west) and allowed 60 s to find the hidden platform. After locating the platform, the animal was allowed to remain on the platform for 10 s. If the animal did not locate the platform within 60 s, it was placed on it for 10 s. Following each trial, the animal was placed in a cage and kept warm with an infrared heating lamp. The inter-trial interval was approximately 4 min. Baseline testing revealed impairment on this task similar to published reports (Gallagher *et al*., 1993;
Gage et al., 1988; Markowska et al., 1989; Rapp and Amaral, 1992). Rats that received an average day 3 latency to platform score >30s were considered impaired and tested separately. Unimpaired aged rats were submitted to anesthetic treatment, further behavioral testing and hippocampal protein analysis. Post-anesthesia behavioral testing was performed to assess anesthesia-induced spatial learning deficits in aged rats. Spatial acquisition learning was assessed at 2 weeks and 3 months post-anesthesia. The hidden platform was placed in the NE (2 week testing) and SW (3 month testing) quadrants. Latency to platform, path length, and swim speeds were measured for each rat. Individual scores were compared to baseline scores.

**Antibodies and immunoblotting**

Mouse monoclonal antibodies against NR2B (1:1000, Cell Signaling Technologies), and β-actin (1:5000, Sigma), polyclonal antibodies against NR2A (1:1000, Cell Signaling Technologies), NR1 (1:1000, Cell Signaling Technologies) Caspase 3 (1:1000 Cell Signaling Technologies) were used. Animals were sacrificed by decapitation immediately following anesthesia, 24h post-anesthesia, and after behavioral testing (2 weeks or 3 months post-anesthesia). Naïve age matched rats were sacrificed at the same times as rats in experimental conditions. The bilateral hippocampi were dissected at 4°C in saline and frozen in liquid nitrogen within 3 min of decapitation, and stored at -80 C. Samples were homogenized in PTN50 extraction buffer (50 mM NaPi, pH7.4, 50mM NaCl, and 1% Triton X-100) with protease and phosphatase inhibitors (Sigma-Aldrich). Samples were spun at 12,000 x g for 3 min, and were taken from the supernatant, avoiding both the pellet and the lipids on the surface. Laemmli sample buffer was added. Proteins were resolved in 10–20%, or 10% Tris-HCl Criterion pre-casted gels
(Bio-Rad, Hercules, CA), transferred to polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA), placed in blocking buffer (PBS, 0.1% Tween 20, and 0.4% I-Block (Applied Biosystems)), then membranes were incubated for 1h with primary antibodies followed by appropriate secondary HRP-linked antibodies (Cell Signaling Technology). Visualization of signal was enhanced by chemiluminescence using a phototope-HRP detection kit (Cell Signaling Technology). To control for protein loading, immunoblots were stripped with Restore Western blot stripping buffer (Pierce, Rockford, IL), and blotted with monoclonal anti-β-actin antibody (1:5000; Sigma). Quantification of band density was performed using the NIH ImageJ 1.34 software, and data were normalized to β-actin.

**Ro 25-6981 Treatment**

Ro 25-6981 (molecular formula: C_{22}H_{29}NO_2·HCl, Sigma) was diluted in saline vehicle and administered (1 mg/kg) by intraperitoneal injection (IP) 15 min prior to preparation for general anesthesia. This dosing and treatment was chosen based on therapeutic success of previous studies in our laboratory (Bigford *et al.*, 2009). For vehicle treated rats, saline was administered by IP injection as described above. Eighteen-month-old rats were behaviorally pretested as described above. Healthy, unimpaired aged rats (N=20) were randomly assigned to one of the following groups: Anesth-Ro (Ro pretreatment followed by 4h anesthesia treatment), Anesth-Veh (vehicle pretreatment with 4h of anesthesia treatment), N-Ro (Ro pretreatment with no anesthesia), N-Veh (vehicle treatment with no anesthesia) (N=5/group). Rats were injected 15min before preparation, which took 30 min, followed by 4h anesthesia treatment, or no anesthesia and then sacrificed by decapitation (total time 4h 45min). Naïve rats were injected with
Ro or saline, kept in the same room and sacrificed at the same time point as anesthesia treated rats.

**Perfusion Fixation**

Animals were re-anesthetized with 3% isoflurane nitrous oxide/oxygen mixture and perfused with cold saline (2min, 75mL) followed by 4% paraformaldehyde (300mL) solution, and the brains were removed and placed in 4% paraformaldehyde at 4°C for 3 days. Brains were then blocked and embedded in paraffin; transverse tissue sections 10 µm thick were taken at 300-µm intervals.

**Neuronal cell counts**

Alternate sections representing levels within the hippocampus (bregma levels -2.8 to -4.8) were immunostained for NeuN, a neuronal marker (Chemicon International, Temecula, CA) using diaminobenzidine (DAB) as the chromophore. Briefly, sections (10 µm thick) were deparaffinized, rehydrated, and incubated overnight at 4°C with NeuN (1:500). After washing, with 0.1 M PBS pH 7.4 with 0.4% Triton, secondary antibody mouse IgG antibody was applied for 90 min at room temperature. After further washing, ABC Elite was applied for 90 min, slides were rinsed with PBS followed by Acetate-Imidoasole Buffer (pH 7.2), and then reacted with NiDAB (2.5% Nickel Ammonium Sulfate Acetate-Imidasole buffer. The number of NeuN positive cells was quantified in the hippocampus regions CA1, CA3, and dentate gyrus to determine cell loss as a result of prolonged anesthesia exposure at 3 months post-anesthesia. Serial vibratome sections (10µm) of the rat brain at the level of the hippocampus were divided into 5 groups. Each contained 10 sections representing the hippocampus. The hippocampal regions: CA1, CA3 and the dentate gyrus in anesthesia treated and naïve rats (at 3 months post-
anesthesia treatment) were analyzed using an Axiophot (Zeiss, Thornwood, NY) research microscope, furnished with a fully motorized 3-D LEP stage, Optronix cooled video camera, and Stereo-Investigator software package (MicroBrightfield, Inc., Colchester, VT). To perform cell number estimation in the structure volume, the optical fractionator method and optical dissector probe were used. Dimensional analysis of the optical dissector was designed based upon the cell distribution system on the section, and optical fractionator grid size (56 x 170µm) was determined based on the results of the preliminary count of the naïve brain sample to allow 200 counts per section in the hippocampal region. CA1, CA3, and the dentate gyrus were analyzed separately, with a sum of these regions representing the whole hippocampus. Immunoreactive cells were those that had degrees of staining greater than background. Data were expressed as numbers of NeuN-positive cells in the regions of the hippocampus at specified Bregma levels (-2.8mm, -3.3mm, -3.8mm, -4.3mm and -4.8mm) and for all sections summed.

**Statistical analysis**

Healthy, unimpaired animals were randomized to experimental and control groups. Data are expressed as mean +/-SEM. Statistical comparisons between experimental and control groups were made using one-way ANOVA with Tukey’s multiple comparisons post-hoc analysis, or repeated measures analysis of variance depending on outcome measure. P-values for significance used were P<0.05.
Results

Aging induces an increase in caspase-3 cleavage

To determine caspase-3 activation levels of aged Fischer 344 following isoflurane anesthetic exposure, animals were killed by decapitation immediately, 2 weeks or 3 months post-anesthesia. Hippocampal and cortical tissues from experimental groups were subjected to western blot analysis with antibodies against caspase-3. Representative blots for hippocampus and cortex are shown. In the hippocampus, caspase-3 activation, measured by caspase-3 fragment (~17kD) quantification, was elevated in naïve rats at 21 months compared to 18 months (p=0.0036, Fig.4.1B). No significant differences were detected in the cortex. Additionally, no significant differences were detected in caspase 3 activation between anesthesia treated and naïve rats.

Anesthesia treated rats display spatial learning deficits at 3 months post-anesthesia

In Figure 4.2, anesthesia treated rats displayed significantly higher mean path length (p=0.003) and day 3 latencies (p=0.0043) versus naïve age-matched rats at 3 months post-anesthesia. Additionally, anesthesia treated rats demonstrated higher mean path length at 3 months post-anesthesia compared to baseline (p=<0.001, Fig. 4.2B). From 2 weeks to 3 months the mean path length increased significantly (p=0.013, Fig. 4.2B) for anesthesia treated rats compared to naïve. Swim speeds were similar between groups (data not shown). Overall, we showed that aged rats exposed to 4h 1.8% isoflurane N₂O/O₂ mixture anesthesia exhibited deficits in spatial learning acquisition at 3 months post-anesthesia.
Isoflurane anesthesia induces an increase in NMDA receptor subunit NR2B protein expression in the hippocampus and cortex of aged rats

In Figure 4.3, hippocampal and cortical brain samples from 18-month-old rats were immunoblotted for NMDA receptor subunits to determine changes in protein expression as a result of anesthesia exposure immediately following anesthesia, 2 weeks and 3 months post-anesthesia compared to naïve controls. No differences were detected between anesthesia and naïve groups for protein expression of NR2A in the hippocampus or cortex. An increase in NR2A protein expression was demonstrated in the hippocampus between naïve rats at 18 months and 21 months (p<0.001, Fig. 4.3A). For NR2B, rats treated with anesthesia displayed higher protein expression in the hippocampus and the cortex at 21 months versus naïve rats at 18 months (p=0.003) and 21 months (p=0.006) (Fig. 4.3A). There was also an acute increase in NR2B in the hippocampus and cortex between anesthesia treated and naïve rats at 18 months (p=0.002, p=0.002 Fig 4.3). For NR1 protein levels, an increase in protein expression of NR1 was observed between naïve 18-month-old and 21-month-old aged rats in the hippocampus (p=0.008, Fig. 4.3A) and cortex (p=0.007, Fig. 4.3B), and between naïve at 18 months versus anesthesia treated at 3 months post-anesthesia (p=0.003, Fig. 4.3B), and at 3 months post-anesthesia naïve versus anesthesia-treated (p=0.009). Additionally, NR1 protein levels were increased for anesthesia treated rats at 3 months post-anesthesia compared to naïve 18-month-old rats in the hippocampus (p=0.002, Fig. 4.3A) and cortex (p=0.0039, Fig. 4.3B).
Isoflurane induces a decrease in ERK1/2 activation in the hippocampus and cortex of aged rats

Hippocampal and cortical brain samples from 18-month-old rats anesthesia-treated or non-treated (naïve) were immunoblotted for phospho-ERK1/2 and total ERK1/2 to determine changes in activation of ERK1/2, determined by the ratio of phospho- to total-ERK, following anesthesia exposure. A decrease in phospho-ERK1/2 was detected in anesthesia treated rats compared to naïve rats 3 months post-anesthesia in the hippocampus (pERK1: p=0.004, pERK2: p=0.02, Fig. 4.4A) and cortex (pERK1: p=0.002, pERK2: p=0.001, Fig. 4.4B). Additionally, an age-related decrease was detected in ERK2 activation in the cortex of naïve rats from 18 to 21 months (p=0.011, Fig. 4.4B).

Ro 25-6981 attenuates acute NR2B increase in anesthesia treated aged rats

In Figure 4.6, Ro 25-6981 (Ro) pretreatment reduced hippocampal NR2B protein expression in 18-month-old rats exposed to isoflurane anesthesia treatment. Hippocampal NR2B protein levels were significantly reduced (P=0.008, Fig. 4.6) in the Ro treatment group compared to vehicle. The hippocampal NR2B protein expression of 18-month-old naïve rats pretreated with Ro that received isoflurane were not significantly different (n.s.) from naïve 18-month-old rats.

Discussion

There is extensive literature reporting incidences of cognitive deficits following general anesthesia in humans (Deiner and Silverstein, 2009) and rodent models (Culley et al, 2003; 2004), with age representing the greatest risk factor for the development of POCD (Canet et al., 2003; Johnson et al., 2002; Monk et al., 2008; Newman et al., 2007). Additionally, detection of POCD occurs within weeks following anesthetic
exposure and manifests as chronic effects on the cognitive performance of patients. There may be an interaction of advancing age and anesthetic insult that results in cognitive dysfunction for some elderly patients. To identify protein expression changes in caspase-3 fragment and NMDA receptor subunits, we investigated the effect of anesthesia exposure on the aged brain in vivo. We also investigated spatial learning deficits in aged Fischer 344 rats at early (2 weeks) and late (3 months) time points post-anesthesia. Our findings identified isoflurane-induced increases in hippocampal and cortical NR2B protein expression at acute and chronic time points corresponding to poor behavioral performance.

Previous researchers demonstrated isoflurane-induced increases in caspase 3 activation in vitro at early time points following isoflurane exposure (Xie et al., 2006; 2008; Zhang et al., 2009). In contrast, no change was detected in activated caspase-3 between groups in the hippocampus or cortex in vivo (Figure 4.1). There was an age-related increase in cleaved caspase-3 between 18- and 21-month-old rats regardless of anesthesia treatment. It is possible caspase-3 occurs at early time points similar to the findings demonstrated in culured neurons (Xie et al., 2006; 2008; Zhang et al., 2009). Additionally, there may be two separate mechanisms involving caspase 3 activation in culture systems versus in the organism during and following anesthetic exposure. It is also important to consider that isoflurane exposure to cultured cells is a much more severe insult, with cell death being the ultimate outcome (Zhang et al., 2009). In an organism, such as rodent or human exposed to anesthesia, the brain may not endure the same intensity of cellular injury. The amount of isoflurane that reaches the brain is measured by the partial pressure of isoflurane in arterial blood, which is 50-60% of the
concentration in humans (Kennedy and Longnecker, 1996) and rats (Holdcroft et al., 1999). In an organism treated with isoflurane anesthesia, neurons are exposed to much less of the anesthetic agent than neurons in culture, which may explain the conflicting findings between previous studies performed in vitro and the results presented here.

Behavioral testing of aged Fischer rats revealed anesthesia-induced spatial learning deficits at 3 months post-anesthesia, but not at two weeks (Figure 4.2). Short-term cognitive impairment, common after general anesthesia, is typically attributed to incomplete clearance of the anesthetic (Moller et al., 1993). The delayed onset of cognitive dysfunction in rats mimics the clinical incidence of POCD in elderly surgical patients because these POCD symptoms typically manifest in the weeks and months after surgery, but not immediately following anesthetic exposure. The delayed presence of spatial learning impairments demonstrated here may result in chronic disruption in learning and memory signaling pathways that occurs over time.

We hypothesized that a chronic disruption in NMDA-mediated signaling results from exposure of isoflurane anesthesia in the aged rat brain in vivo. Previously, an increase in NR2B subunit protein expression in the hippocampus of young mice 24h after isoflurane anesthesia treatment was reported (Rammes et al., 2009). We report similar findings here in aged rats, with acute and chronic increases in NR2B protein expression levels in the cortex and hippocampus of anesthesia-treated aged rats (Figure 4.3). Aged rodents with higher levels of NR2B demonstrate poor behavioral outcomes compared to age-matched controls (Zhao et al., 2009). Our findings suggest that the chronic upregulation of NR2B protein levels in the hippocampus and cortex correspond to spatial learning deficits observed in anesthesia-treated aged rats. One explanation may involve a
change in NR2B subunit protein expression shift to more extra-synaptic localization of NR2B subunit, with an increase in LTD (Massey et al., 2004) and/or increased inhibition of CREB that interferes with brain-derived neurotrophic factor (BDNF) levels, and results in loss of mitochondrial membrane potential, and cell death (Hardingham et al., 2002). However, we were unable to detect anesthesia-induced cell loss in aged rats 3 months post-anesthesia (Figure 4.4).

Also, the chronic upregulation of NR2B may result in disruption of downstream learning and memory signaling pathways. Hippocampal ERK1/2 is required for acquisition of hippocampal-dependent spatial learning tasks, such as MWM (Atkins et al., 1998; Selcher et al., 1999). Here we demonstrate a chronic reduction in phospho-ERK1/2 levels in the hippocampus and cortex of anesthesia treated aged rats (Figure 4.5), which exhibited anesthesia-induced chronic spatial learning deficits at 3 months post-anesthesia. Protein expression levels of NR2B upregulation has been linked to reduction in activated ERK1/2 protein levels (Kim et al., 2005). Therefore, anesthesia-induced changes in NR2B protein levels in the hippocampus and cortex may initiate dysfunction in NMDAR-ERK mediated learning and memory signaling pathways, resulting in chronic spatial learning deficits in anesthesia treated aged rats.

In a separate experiment, we attenuated the anesthesia-induced NR2B specific elevation in the hippocampus of aged rats by pre-treating with the selective NR2B antagonist, Ro 25-6981 (Ro, Figure 4.6) Ro is the most potent and selective blocker of NR2B-containing NMDA receptors, with high blocking potency for NMDA receptors in electrophysiological experiments in vitro (Fischer et al., 1997) and ability to inhibit binding of 125I-MK801 (iodo-(+)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclohept-
5, 10-imine maleate) to receptors made from NR1/NR2B but not NR1/NR2A (Lynch et al., 2001). The therapeutic capability of Ro was previously demonstrated by successfully preventing over-activation of NMDA receptors in animal models of traumatic brain injury (Bigford et al., 2010), Parkinson’s disease (Loschmann et al., 2004), and neuropathic pain (Chizh et al., 2005). Chronic antagonism of NMDA receptors results in upregulation of the receptor in vitro (Gunduz-Bruce, 2009). Chronic antagonism of NMDA receptors by isoflurane during anesthetic exposure may result in the observed increase in NR2B protein expression. Ro pretreatment inhibited acute upregulation of NR2B in the hippocampus.

Limitations of the current study include the variability of spatial learning performance of aged rats. Eighteen-month old Fischer rats that exhibited pre-existing spatial learning deficits were tested separately (Figures 4.7 and 4.8). Detection of anesthesia induced spatial learning deficits on already impaired rats may be difficult due to floor effects. A more discriminating test of spatial learning is required to determine anesthesia-induced impairments in these animals. Our results were summarized for the subgroup of healthy, unimpaired rats and therefore cannot be extrapolated to aged Fischer rats in general. Additionally, the trauma of anesthesia can produce non-specific inflammatory and immune responses that may affect cognitive outcomes. Therefore, the effect of isoflurane anesthesia on NMDA receptors represents only one potential mechanism contributing to the impairment of spatial learning performance in aged rats.

Age-related functional changes in the NR2B subunit of the aged brain produce vulnerabilities leading to post-anesthesia consequences for aged rats. Taken together, our results indicate a role for an elevation in NR2B protein expression following isoflurane
anesthesia in the aged rat brain, corresponding to spatial learning deficits in these animals. We also suggest a therapeutic intervention for inhibition of NR2B protein expression increases in the hippocampus, with Ro 25-6981. Further behavioral studies are required to demonstrate the efficacy of Ro pretreatment for chronic spatial learning impairment in aged rats.
Figures for Chapter IV

Figure 4.1: Age-related increase in caspase 3 activation in the aged rat cortex and hippocampus
Representative immunoblots for (A) hippocampal and (B) cortical protein lysates from the brains of aged rats in the following experimental groups: naïve at 18 months (N (18m)), anesthesia treated at 18 months (An (18m)), anesthesia treated at 2 weeks post-anesthesia (An (+2wk)), naïve at 21 months (N (+3m)), and anesthesia treated at 3 months post-anesthesia (An (+3mo)) for antibodies against caspase 3, and β-actin, followed by quantification of Caspase 3 fragment protein expression. [Data are presented as means, error bars represent standard error mean (s.e.m.), n=4-5/group. One-way ANOVA and Tukey’s post-hoc analysis were performed, *p<0.05, **p<0.005].
Figure 4.2: Spatial learning acquisition water maze performance in aged rats following anesthesia treatment

(A) Latency to platform was measured at three testing points: baseline (days -7 to -5), post-anesthesia testing at 2 weeks (days 15 to 17) and 3 months (days 90 to 92) in anesthesia treated (open circle, dotted line) and naïve (closed square, solid line) aged rats in a 3 day spatial acquisition task via MWM. Each day, four trials were performed. Latency to platform is plotted for the average of each day of testing. (B) Mean path length was determined for anesthesia treated (white bars) and naïve (black bars) aged rats on testing day 3 of the testing points (days -5, 17, and 92). [Latencies to platform and mean path lengths are plotted, error bars represent standard error mean (s.e.m.) Repeated measures of analysis of variance were performed, *p<0.05, **p<0.005, ***p<0.001].
Figure 4.3: Effect of isoflurane anesthesia on acute and chronic protein expression of NMDA receptor subunits in the hippocampus and cortex of aged rats.
(A) Representative immunoblots for hippocampal and (B) cortical protein lysates from the brains of aged rats in the following experimental groups: naïve at 18 months (N (18m)), anesthesia treated at 18 months (An (18m)), anesthesia treated at 2 weeks post-anesthesia (An (+2wk)), naïve at 21 months (N (+3m), and anesthesia treated at 3 months post-anesthesia (An (+3mo)) for antibodies against NR2A, NR2B, NR1 and β-actin, followed by quantification of protein expression. β-actin was used as an internal standard and control for protein loading. [Means are plotted, error bars represent standard error mean (s.e.m.), n=4-5/group. One-way ANOVA and Tukey’s post-hoc analysis were performed, *p<0.01, **p<0.005, ***p<0.001]
Figure 4.4 Anesthesia did not alter the number of NeuN positive cells in the hippocampus of aged rats treated with anesthesia at 3 months post-anesthesia.

Stereological analysis of hippocampal sections stained with NeuN antibody indicates that anesthesia does not reduce the number of NeuN+ cells in hippocampal regions for aged rats treated with anesthesia versus naïve. Perfusion fixation was performed at 3 months post-anesthesia. Bregma levels -2.8, -3.3, -3.8, -4.3, and -4.8 were counted in the three hippocampal regions: (A) CA1, (B) CA3, (C) dentate gyrus, and (D) the total hippocampus. (E) NeuN+ cells were totaled for the regions. No statistical significance between groups was detected. [Means are plotted, error bars represent SEM, n=4-5/group. Repeated measures ANOVA and Tukey’s post-hoc analysis were performed].
Figure 4.5 Effect of isoflurane anesthesia on ERK1/2 activation in the hippocampus and cortex of aged rats

(A) Representative immunoblots for hippocampal and (B) cortical protein lysates aged rats in the following experimental groups: Naïve at 18 months (N (18m)), anesthesia treated at 18 months (An (18m)), anesthesia treated at 2 weeks post-anesthesia (An (+2wk)), naïve at 21 months (N (+3m)), and anesthesia treated at 3 months post-anesthesia (An (+3mo)) for antibodies against phospho-ERK1/2 and ERK1/2, followed by quantification of the ratio of phospho-ERK over total ERK to determine ERK activation. [Means are plotted, error bars represent standard error mean (s.e.m.), n=4-5/group. One-way ANOVA and Tukey’s post-hoc analysis were performed better learners and poor learners separately, #p<0.01, *p<0.05, ##p<0.005, **p<0.001].
Ro 25-6981 Treatment attenuated an anesthesia induced-increase in NR2B levels in the hippocampus of 18-month-old rats

Representative immunoblots for antibodies against NR2B in hippocampal protein lysates from brains of rats that received isoflurane anesthesia treatment or naïve rats that received no anesthesia with Ro 25-6981 or vehicle pretreatment. β-actin was used as internal standard and control for protein loading. Ro pretreatment significantly (p=0.03) reduced hippocampal NR2B protein levels to baseline levels. Ro did not alter protein expression levels of NR2B in rats that received no anesthesia. [Means are plotted, error bars represent standard error mean (s.e.m.), n=5-7/group. One-way ANOVA and Tukey’s post-hoc analysis were performed, *p<0.05].
Figure 4.7 Spatial acquisition learning for impaired rats before and after prolonged isoflurane anesthesia exposure.

Previously, researchers demonstrated spatial learning impairment in anesthesia treated aged but not young rats compared to naïve age-matched controls (Culley et al., 2003; 2004; Crosby et al., 2004). Eighteen-month-old rats were divided based on baseline performance before anesthesia exposure. Pre-anesthesia impairment was determined by a day 3 average latency score >30s. Impaired rats were randomized to receive isoflurane anesthesia treatment (4h, 1.8% isoflurane nitrous oxide/oxygen mixture anesthesia or no anesthesia ( naïve), then tested in a 3 day spatial acquisition task via MWM at 2 weeks (days 7-9) and 3 months (days 90-92) post-anesthesia treatment. There were no significant differences in mean path length between anesthesia (white bars) and naïve (black bars) rats at baseline, 2 weeks or 3 months post-anesthesia in the impaired groups tested. [Means are plotted, error bars represent standard error mean (s.e.m.), n=12-15/group. One-way ANOVA and Tukey’s post-hoc analysis were performed].
Figure 4.8 Age related caspase 3 activation was detected in aged impaired Fischer 344 rats. Hippocampal protein lysates from the brains of aged rats (impaired prior to anesthesia exposure) were immunoblotted with antibodies for (A) caspase 3, (C) NR2B, and (E) NR1. Cortical lysates were also analyzed for (B) caspase 3, (D) NR2B and (F) NR1. Quantification of immunoblots are presented. Naïve impaired rats at 21 months demonstrated elevated levels of caspase 3 fragment in the hippocampus (p=0.036) and cortex (p=0.029) compared to naïve 18 month old controls. Anesthesia treated impaired rats at 3 months anesthesia, also demonstrated elevated levels of caspase 3 activation in the cortex (p=0.04). [Means are plotted, error bars SEM, n=4-5/group. One-way ANOVA and Tukey’s post-hoc analysis were performed, *p<0.05].
Chapter V

DISCUSSION

Summary

My studies have identified several underlying mechanisms of age-related cognitive deficits that result in the aged brain being susceptible to spatial learning impairment following anesthesia treatment. First, in aged rats brains the NLRP1 inflammasome demonstrates elevated activation corresponding to age-related cognitive deficits in a spatial learning task (Chapter II). Second, anti-inflammatory treatment improved spatial learning performance in aged rats via inhibition of NLRP1 inflammasome components (Chapter II). Third, isoflurane induced caspase-3 activation and NR2B protein expression increases in neuronal cultures, while cell death was attenuated by Ro 25-6981, an NR2B specific antagonist, suggesting involvement of NR2B-containing NMDA receptors in isoflurane-induced cell death (Chapter III). Fourth, isoflurane anesthesia induced spatial learning impairments in age rats previously unimpaired on a spatial learning task via an increase in NMDA receptor NR2B subunit in hippocampus and cortex (Chapter IV). The acute isoflurane-induced increase in NR2B protein expression in the hippocampus of aged rats was attenuated by pretreatment with the NR2B blocker, Ro 25-6981 (Chapter IV). In addition, isoflurane anesthesia induced an increase in mortality rate in aged rats that mimics that of the clinical population. In summary, these studies have discovered NLRP1 inflammasome activation in the aged brain, and introduced a novel mechanism of anesthesia-induced spatial learning deficits in aged rats involving the NMDA receptor subunit NR2B.
These studies have addressed questions that impact on the fields of aging, inflammation, anesthesia, and learning and memory. Given the current trends in population growth for individuals over 65, age-related investigations are of great importance to researchers, anesthesiologists and other clinicians. Investigations of the interaction between advanced age and anesthesia exposure are also especially important when one considers the number of elderly surgical patients who face an elevated risk of developing anesthesia-related cognitive decline. The discovery of novel targets for the pharmacological treatment of age-related cognitive decline and POCD would provide invaluable tools for basic scientists and medical professionals, while potentially offering improved cognitive health for the growing elderly population.

The primary aim of my studies focused on anesthesia-induced spatial learning deficits in aged rats. However, it was necessary to identify specific vulnerabilities in the aged brain that may impart susceptibility of environmental stressors, like anesthesia, in order to fully comprehend the impact of such insults. It was reasoned that anesthetic exposure might exacerbate or hasten normal mechanisms of cognitive aging, leading to cognitive impairments. My discovery of inflammasome activation in the aged brain was somewhat serendipitous during my original investigation, in that cognitive deficits were discovered in aged animals prior to anesthetic exposure. Upon identification of inflammatory changes in the brains of aged rats, a tangential line of research was explored investigating the involvement of the NLRP1 inflammasome in cognitive deficits present in aged animals before anesthetic exposure. I also continued along my main line of research in the investigation of anesthesia-induced spatial learning impairments in aged rats via an increase in NMDA receptor subunit NR2B protein expression in the
hippocampus and cortex. This finding ultimately led to the formation of a proposed mechanism of POCD in aged rats presented in this chapter.

**Age-related cognitive decline**

The hippocampus is typically chosen by researchers to investigate age-related cognitive decline. The hippocampus demonstrates structural and physiological changes as a result of normal aging that may contribute to cognitive deficits (Barnes and McNaughton, 1980; Driscoll *et al.*, 2009; Foster *et al.*, 1991; Geinisman *et al.*, 1992; 2004; Kennedy *et al.*, 2009; Raz *et al.*, 2005; Rosensweig and Barnes, 2003; West, 1993). Previous studies found the Morris water maze to be reliably sensitive to age-related impairments in the hippocampus (Fischer *et al.*, 1992; Gage *et al.*, 1984; Gallagher *et al.*, 1989; 1993). Therefore, I chose a hippocampal-dependent spatial learning acquisition task via Morris water maze as the behavioral paradigm to study the effects of aging and anesthesia.

In my investigation of anesthesia induced spatial learning deficits in aged rats, it was necessary to perform behavioral testing of aged animals to determine pre-existing spatial learning impairments in rats and classify them as such. Historically, researchers have demonstrated striking individual differences in spatial learning performance of aged rats (Gage *et al.*, 1984; Gallagher and Burwell, 1989; 1993; Rapp *et al.*, 1987). Initial testing of spatial learning performance included young (3-month-old) and aged (18-month-old) male Fischer 344 rats. Animals were tested in a 3-day spatial acquisition task as previously described (Chapter IV). Analysis of latency scores revealed a bimodal distribution of the scores for aged rats (see Appendix A Figure 6.1). During evaluation of the performance of aged rats, it became apparent that aged animals fell into one of two
categories: impaired or unimpaired. Unimpaired rats performed similarly to their younger counter parts, whereas impaired rats performed poorly on spatial learning tasks (see Appendix A Figure 6.2). These groups have been previously described in the literature (Fischer et al., 1989, 1992; Frick et al., 1995). Based on my findings and previous reports, it appears that some aged rats become less proficient in learning the information that is required for spatial navigation to a specific location, and demonstrate age-related spatial learning impairments. Based on my initial behavioral findings, age-related cognitive deficits became a secondary focus of my research.

**The NLRP1 Inflammasome**

Much of the aging research centers on the role of inflammatory processes in age-related cognitive decline. Age-related inflammation is associated with several neurodegenerative diseases, yet even in the absence of neurological disease, the aged brain exhibits a heightened inflammatory profile (Lee et al., 2000). Recent evidence demonstrates a role for NLRP1 inflammasome activation following CNS injury (Abulafia et al., 2009; de Vaccari Rivero et al., 2008, 2009; Silverman et al., 2009). Despite extensive evidence of age-related elevations in inflammatory processes, investigations of inflammasome activation in the aged brain were lacking. Based on the elevated inflammatory profile of the aged brain and recent evidence of NLRP1 inflammasome activation in the brain, I hypothesized that NLRP1 inflammasome activation may contribute to age-related spatial learning deficits.

In Chapter II, it was shown that levels of the pro-inflammatory cytokines, IL-1β and IL-18, are elevated in the aged rat hippocampus. The finding of age-related elevations in IL-1β is supported in the literature. There is an increase in IL-1β in the
brain and in microglia activation of neurologically intact aging patients (Ershler et al., 1993; Rubenoff et al., 1998; Wilson et al., 2002). Additionally, in Chapter II, some NLRP1 inflammasome components were elevated in the aged brain leading to heightened NLRP1 inflammasome activation producing an increase in the secretion of IL-1β and IL-18. Specifically, caspase-1, caspase-11, XIAP, the X-linked inhibitor of apoptosis, P2X7 receptor, and Pannexin-1, the pore-forming protein, were shown to be elevated in the aged brain compared to younger animals. Upon activation of the NLRP1 inflammasome, caspase-1 and caspase-11 are activated to promote rapid cleavage of pro-IL1β and IL-18. The data show an elevated level of these caspases in the aged hippocampus suggesting an increase in inflammasome activity in the aged brain. Additionally, cleavage of XIAP, suggested to be the putative inhibitor of apoptosis in the NLRP1 inflammasome (de Rivero Vaccari et al., 2008, 2009; Silverman et al., 2009), was associated with aging. XIAP cleavage into fragments reduces the proteins inhibitory effect on caspases (Katz et al., 2001; Keane et al., 2001; Lotocki and Keane, 2002) that may increase activation of caspase-1, leading to increased processing of IL-1β and IL-18. Furthermore, age-related increases in the pore-forming protein Pannexin-1 and P2X7 receptor protein expression were observed. Pannexin-1 is involved in transport of extracellular K+ ions in neurons and astrocytes to stimulate the NLRP1 inflammasome, following binding of ATP to P2X7 receptors. Therefore, increases in Pannexin-1 and P2X7 receptor in the aged hippocampus suggest a facilitation of K+ influx and subsequent NLRP1 inflammasome activation. These studies did not reveal an age-related change in the scaffolding protein, NLRP1, or the adaptor protein, ASC. However, the proportions of changes observed in other NLRP1 inflammasome proteins indicate increased activation of the complex in the
aged brain. These findings are supported by previous investigations of inflammasome activation following acute injury, which have demonstrated a lack of change in NLRP1 protein expression (de Rivero Vaccari et al., 2008, 2009). Taken together, these findings suggest that there is heightened activation of the NLRP1 inflammasome in the aged brain that may contribute to increased levels of proinflammatory cytokines, IL-1β and IL-18.

Inflammatory cytokines disrupt normal physiology and contribute to age-related deficits in cognitive function (Lynch, 2002; Pugh et al., 2001; Yirmiya et al., 2002). Activation of the NLRP1 inflammasome initiates processing of IL-1β and IL-18 (Martinon et al., 2002; Martinon and Tschopp, 2004; Miao et al., 2006; Ozoren et al., 2006). The pro-(inactive) forms of the cytokines are stored in the cytosol. Upon activation, IL-1β and IL-18 are released from the cell. The high affinity IL-1 type 1 receptor, which binds IL-1β (Dinarello, 1997, 1998; Rothwell et al., 1997), is constitutively expressed in the hippocampus (Benveniste et al., 1995; Farrar et al., 1987; Rothwell and Hopkins, 1995). The pathological effects of elevated levels of IL-1β can be particularly detrimental to hippocampal-dependent tasks, which was demonstrated by my finding of IL-1β elevation and corresponding spatial learning deficits in aged rats. In agreement with this finding, IL-1β impairs hippocampal-dependent memory tasks such as contextual fear conditioning (Barrientos et al., 2002; Maier and Watkins, 1995; Pugh et al., 1999, 2001) and Morris water maze tasks (Pugh et al., 1998; Yirmiya et al., 2002). IL-1β has also been shown to play a role in disruption of neuronal functions involved in synaptic plasticity in the hippocampus, such as LTP, neurogenesis and neurite outgrowth (Ekdahl et al., 2003; Li et al., 1997; Lynch et al., 2004; Monje et al., 2003; Neumann et al., 2002; Vereker et al., 2000). It is possible that activation of the NLRP1 inflammasome
is maintained at a higher level and/or has a lower threshold of activation in the aged brain. This elevation in inflammasome activity results in potential pathologic levels of IL-1β that may disrupt various neuronal functions involved in synaptic plasticity in the hippocampus. My findings suggest that increased IL-1β levels observed with age may underlie age-related cognitive impairments in hippocampal-dependent learning and memory. Therefore, it is possible that an anti-inflammatory treatment, aimed at reducing IL-1β levels in the aged brain may improve performance of aged rats on hippocampal-dependent tasks.

In Chapter II, treatment with probenecid, a drug commonly used in the treatment of gout and recently identified as an inhibitor of inflammasome activation (Silverman et al., 2009), reduced protein expression of P2X7 receptor and Pannexin 1. Reduction of these protein levels was thought to reduce NLRP1 inflammasome activation in the aged brain. The resultant reduction in activation of caspase-1 further verified inhibition of NLRP1 inflammasome activation by probenecid. Probenecid-treated rats demonstrated superior spatial learning performance compared to vehicle-treated age-matched controls. Caspase-1 activation cleaves pro-IL-1β to produce the active form and an increase in IL-1β is implicated in the decline of synaptic plasticity and impaired performance on cognitive tasks such as contextual fear conditioning and Morris water maze (Godbout et al., 2005; Godbout and Johnson, 2009; Heyser et al., 1997; Monje et al., 2003; Vallieres et al., 2002; Vereker et al., 2000). Therefore, the probenecid-induced reduction in caspase-1 activation and levels of IL-1β may help explain the observed improvement in spatial learning performance in treated animals compared to age-matched controls. Previous reports have also shown that anti-inflammatory treatments, which reduce IL-1β
levels in the aged brain, improve cognitive behavioral outcomes in aged rodents (Gemma et al., 2005; Mesches et al., 2004). Treatment with sulindac, a non-steroidal anti-inflammatory drug, reduced IL-1β levels and reversed learning and memory deficits in aged rats (Mesches et al., 2004). Additionally, the irreversible caspase-1 inhibitor Ac-YVAD-CMK was used to block caspase-1 activity, thereby inhibiting IL-1β processing and maturation, ameliorating age-related memory deficits in contextual fear learning (Gemma et al., 2005). Gemma and colleagues (2005) were the first to provide evidence for the involvement of caspase-1 in age-related deficits in a hippocampal-dependent memory task. My data further corroborate the involvement of caspase-1 activity in IL-1β elevation in the hippocampus and corresponding cognitive impairments in aged animals.

Due to the role of IL-1β as a main promoter of the inflammatory cascade (Dinarello, 2000), it is possible that downstream effects of an exacerbated inflammatory immune response may also contribute to learning and memory deficits in aged rats. Since IL-1β stimulates the production of TNFα and IL-6, while also decreasing levels of the anti-inflammatory cytokine, IL-10, which in turn decreases IL-6 (Ye and Johnson, 2001), reduction in IL-1β may be further instrumental in modulating these downstream effectors. The cumulative effect of reducing the inflammatory response may be the mechanism by which learning impairment is reversed in the aged brain. By inhibiting NLRP1 inflammasome activity in the aged brain, caspase-1 activity and processing of IL-1β was reduced. The prevention of cognitive deficits by targeting the pannexin1 channel via probenecid treatment establishes a specific upstream therapeutic target to reduce the inflammatory cascade involving caspase-1 and IL-1β.
There is also a possibility that the reduction in P2X7R and pannexin1 channel with the treatment of probenecid has positive side effects for learning and memory processes involving calcium regulation through astrocytes. Although, my findings suggested a role for neuronal P2X7R and pannexin1 in age-related cognitive decline, these proteins are found abundantly expressed in astrocytes (Burnstock, 2008). Activation of the P2X family of receptors results in an increase in intracellular Ca\(^{2+}\) in astrocytes (Verkhratsky and Kettenmann, 1996; James and Butt, 2002). Reports indicate that the aging process impacts astrocyte physiology such that aging induces larger and more frequent ATP-induced Ca\(^{2+}\) responses (Lin et al., 2007) and changes in purinergic receptor-mediated intracellular Ca\(^{2+}\) levels (Wu et al., 2007). Because the P2X7R and Pannexin1 complex are involved in the signal relay transmission of intercellular calcium waves (Anderson and Nedergaard, 2003; Anderson et al., 2004; Duan et al., 2003; Suadicani et al., 2006), it is possible that aging may induce dysregulation of calcium wave signaling, diminishing the astrocytic function of maintenance and protection of the brain. It is also possible that treatment with probenecid somehow reduced the dysregulation of calcium in astrocytes of the aged rat brain, leading to the observed improvements in spatial learning.

The exacerbated inflammatory response in the aged brain is likely multifactorial. Although Chapter II demonstrated the involvement of NLRP1 inflammasome in age-related cognitive decline, it is likely that IL-1\(\beta\), a secreted cytokine, is produced by various other mechanisms. Increased microglial activity, present in the aged brain (Frank et al., 2005; Godbout et al., 2005; Morgan et al., 1999; Perry et al., 1993), may also contribute to elevated levels of IL-1\(\beta\) and other pro-inflammatory cytokines.
Additionally, infection and illness, which have been linked to cognitive decline in the elderly (Jackson et al., 2004; Janssens and Krauss, 2004; Mulsant et al., 1999; Rockwood et al., 1999), may also contribute to elevated levels of IL-1β and other inflammatory cytokines. The positive feedback machinery of the inflammatory system may also drive the propagation of inflammatory signals within the CNS that contribute to age-related cognitive decline. However, by determining an initiation point of chronic inflammation in the aged brain, like NLRP1 inflammasome over-activation, it is possible to explore specific therapeutic inflammatory treatments for age-related cognitive decline. In Chapter IV, it was suggested that probenecid, an inhibitor of inflammasome activation, might provide treatment for cognitive decline in the elderly. Additionally, treatments aimed at reducing pathologic levels of IL-1β may also result in behavioral improvements in learning and memory processes in the elderly.

**Inflammation and NMDA receptors in age-related cognitive decline**

Age-related cognitive decline is clearly multifactorial. Both the heightened inflammatory profile of the aged brain and age-related alterations in NMDA receptors contribute to deterioration in learning and memory processes during normal aging. Thus far, age-related cognitive decline involving inflammatory molecules, IL-1β in particular, have been discussed. However, various reports have linked age-related increases in proinflammatory cytokines and altered NMDA receptors in the aged hippocampus (Lynch, 1999; McGahon et al., 1999; Murray et al., 1997). Proinflammatory cytokines, such as IL-1β may contribute to NMDA-mediated cognitive deficits in the aged brain, particularly deficits in LTP, synaptic plasticity, and performance on learning and memory tasks. Additionally, IL-1β may be involved in glutamate excitotoxicity (Relton and
Rothwell, 1992; Lawrence et al., 1998; Stroemer and Rothwell, 1998; Pearson et al., 1999; Allan et al., 2000; Jander et al., 2000) that contributes to neurodegenerative diseases (Beal, 1992; Coyle and Puttfarcken, 1993; Lipton and Rosenberg, 1994; Meldrum and Garthwaite, 1990; Nakao and Brundin, 1998). Hence, it is attractive to speculate on the interplay between IL-1β and NMDA receptor function in the aged brain that may impart vulnerability to extrinsic insults to the CNS.

Despite its more accepted role in the immune responses, physiologic levels of IL-1 may be important in modulating neuronal activity in the hippocampus. The role of endogenous IL-1 has been studied in models of LTP. Because NMDA receptors are critical in induction and maintenance of LTP (reviewed in Bliss and Collingridge, 1993), there is evidence to suggest that IL-1β may modulate NMDA receptor function (Balschun et al., 2003; Schneider et al., 1998). Interestingly, IL-1β gene expression is significantly elevated following induction of LTP in the rat hippocampus in situ (Schneider et al., 1998) and in vivo (Balschun et al., 2003). LTP maintenance can be prevented by exogenous IL-1ra (the specific IL-1 receptor antagonist) in Schaffer collaterals in vitro and in the perforant pathway in vivo (Schneider et al., 1998). Additionally, the blockade of IL-1 receptors with IL-1ra results in reversible impairment of LTP maintenance (Schneider et al., 1998). Furthermore, elevated levels of IL-1β, present in the hippocampus of aged rats (Gemma et al., 2002; Lynch, 1999), are implicated in LTP impairment (Murray and Lynch, 1998; Lie et al., 1997; Lynch et al., 2004; Vereker et al., 2000) and cognitive deficits (Gibertini et al., 1995; Godbout et al., 2005; Heyser et al., 1997; Monje et al., 2003; Vallieres et al., 2002; Vereker et al., 2000).
IL-1β is involved in the pathology of several neurodegenerative disorders (reviewed in Lynch, 2002). In that context, it has been suggested that IL-1β is a modulator of the glutaminergic response (Fogal and Hewett, 2008), based on evidence that IL-1β is found in excess in injured tissues in which high levels of glutamate are also found (Liu et al., 2008; Pearson et al., 1999; Vezzani et al., 1999). Additional evidence implicates IL-1β and glutamate excitotoxicity (Allan et al., 2000; Jander et al., 2000; Pearson et al., 1999). Specifically, NMDA-induced excitotoxicity increases IL-1β expression (Pearson et al., 1999). Also, NMDA receptor antagonists suppress IL-1β expression following excitotoxic neuronal damage (Jander et al., 2000). Furthermore, injection of IL-1β enhances excitotoxic neuronal injury in the rat brain (Allan et al., 2000). Finally, IL-1β induces the production of various molecules associated with glutamate toxicity that are implicated in neurological disorders, such as nitric oxide synthase (NOS) and arachidonic acid (AA) (Dayton and Major, 1996; McCann et al., 1998; Sung et al., 2004). Taken together, IL-1β is implicated in glutamate excitotoxicity and the detrimental effects that follow, providing further evidence for an interaction between IL-1 and NMDA receptor signaling.

The most compelling evidence for an interaction between IL-1β and NMDA receptor function can be found in labeling studies that demonstrate colocalization of IL-1 receptors with NMDA receptors (Gardoni et al., 2011; Guo et al., 2007; Viviani et al., 2003; Wei et al., 2008). Researchers have shown that the IL-1 receptor colocalizes with NMDA receptors on neurons in the spinal cord in studies of chronic pain (Guo et al., 2007; Wei et al., 2008). The close proximity of the receptor allows for the activation of IL-1 receptors to facilitate NMDA receptor phosphorylation, inducing change in synaptic
strength (Guo et al., 2007; Ren, 2010; Wei et al., 2008). Since there is a high abundance of IL-1 receptors in the hippocampus (Besedovsky and del Rey, 1996; Hass and Schauenstein, 1997; Rothwell and Hopkins, 1995), it is likely that a similar co-localization of IL-1 receptors and NMDA receptors occurs in the hippocampus. In fact, double labeling for NMDA receptor 2A/B subunits and IL-1R1 demonstrated expression of both receptors with a similar distribution pattern in hippocampal neurons (Viviani et al., 2003). More specifically, IL-1 receptor interacts with the NR2B subunit of the NMDA receptor in the hippocampus, demonstrated by co-immunoprecipitation and co-localization of IL-1R and NR2B with the postsynaptic marker, PSD-95 (Gardoni et al., 2011). Thus, again evidence is present supporting the potential for IL-1β to influence NMDA receptor function in the hippocampus. It is possible that IL-1β signaling through the IL-1 receptor results in over-stimulation of NMDA receptors, which has previously been shown to lead to caspase-3 activation and apoptotic cell death (Laabich and Cooper, 2000; Laabich et al., 2000; 2001; Lin et al., 2005). In the aged brain the situation is magnified. The aged brain sustains an elevated level of IL-1β (Gemma et al., 2002; Lynch, 1999) coupled with increased expression of IL-1 receptor in the hippocampus (Lynch, 1999). Therefore, IL-1β mediated IL-1 receptor phosphorylation of extrasynaptic NMDA receptors may be strengthened, leading to increased caspase-3 activation and cell death. There is also the possibility that the over-activation of NMDA receptors is primed in the aged brain, such that the threshold of neuronal damage and death is lowered in the aged hippocampus. Nevertheless, learning and memory signaling pathways are fragile in the aged hippocampus, and neuronal damage is exacerbated following minor insults. Even in the absence of extrinsic insults, normal aging involves cognitive decline and
learning and memory deficits that may be associated with an interplay between IL-1β and NMDA receptors.

Figure 5.1 depicts a potential mechanism of interaction between inflammasome activation, IL-1β, and NMDA receptors. Briefly, heightened inflammasome activation in the aged hippocampus, demonstrated in chapter II, contributes to the age-related elevations in IL-1β in the aged brain via caspase-1 processing of pro-IL-1β to mature IL-1β which is secreted from the cell. IL-1β then binds to one of the abundant IL-1 receptors present in the aged hippocampus. Activation of the IL-1 receptor then phosphorylates a neighboring extrasynaptic NMDA receptor. Due to the reduced uptake of glutamate in the aged hippocampus, extracellular glutamate levels are elevated (Potier et al., 2010; Saransaari and Oja, 1995; Vatassery et al., 1998). This may result in over activation of the NMDA receptor, leading to impairments in LTP and learning and memory processes. Furthermore, IL-1β mediated over-activation of extrasynaptic NMDA receptors, shown to be involved in cell death (Hardingham et al., 2002), may contribute to caspase-3 mediated cell death in neurons, further contributing to learning and memory impairments in the aged brain.

Spatial learning impairment following anesthesia exposure in aged rats

The aged brain exhibits normal cognitive decline and an increased vulnerability to CNS insults, which can result in further cognitive deficits in learning and memory processes. One such insult that has previously been shown to produce spatial learning impairment in aged rats is isoflurane anesthetic exposure (Culley et al., 2004). Further support of anesthesia-induced spatial learning impairment in aged rats was presented in Chapter IV. Specifically, chronic anesthesia-induced spatial learning deficits were
demonstrated in aged rats that were unimpaired on a spatial acquisition task conducted in the Morris water maze before anesthetic exposure. The behavioral finding that aged rats display anesthesia-induced cognitive deficits is supported in the literature. Anesthesia treatment has differential effects on behavioral outcomes depending on the age of rodents at the time of exposure. Culley and colleagues (2003, 2004) were the first to demonstrate age-specific impairments in rodents following anesthesia exposure. Specifically, at 2 months post-anesthesia, 18-month-old rats demonstrated isoflurane-induced spatial learning impairments on working and reference memory in the 12-arm RAM tasks, while younger (3-month-old) rats did not. In contrast, researchers have demonstrated isoflurane-induced improvements in young rats (Culley et al., 2004) and mice (Rammes et al., 2009). Therefore, age is a significant risk factor for the development of anesthesia-induced spatial learning impairments in rodents. Interestingly, these findings are similar to the clinical population, in that, patients over 65 have a greater risk of developing POCD (Biedler et al., 1999; Moller et al., 2008; Monk et al. 2008; Rasmussen et al., 2001; Steinmetz et al., 2009).

In Chapter IV, aged animals were reported to demonstrate anesthesia-induced spatial learning deficits at three months, but not 2 weeks, post-anesthesia. Specifically, anesthesia-treated aged rats demonstrated higher latency to platform and mean path length measures 92 days after anesthesia compared to naïve age-matched controls (see Figure 4.2), while latency and path length measures were not significantly different between groups on day 14 post-anesthesia. Similar findings have been previously reported (Crosby et al., 2005; Culley et al., 2003, 2004). The delayed onset of anesthesia-induced cognitive impairment in rats mimics the manifestation of POCD in the clinical
population. The number of elderly surgical patients presenting with POCD at hospital discharge is similar to other age groups and controls. Whereas, patients over 60 years of age have a greater risk of developing POCD up to 3 months post-anesthesia (Bedford, 1955; Moller et al., 1998; Monk et al., 2008; Steinmetz et al., 2009). The observed delayed onset of spatial learning deficits in aged rats following anesthesia may involve a build up or summation of factors over time that contributes to dysfunction in learning and memory processes.

**Anesthesia-induced increase in mortality**

General anesthesia exposure has been associated with increased mortality rates among middle-aged and elderly surgical patients (Monk et al., 2008; Weldon et al., 2002). In my studies, aged rats exposed to isoflurane anesthesia had an increased mortality rate compared to age-matched naïve controls (See Appendix A Figure 6.3 for mortality rates and survival curves). Specifically, anesthesia-treated impaired aged rats had a 50% mortality rate, while naïve impaired rats had a 35% mortality rate. The mortality rate for unimpaired rats was also higher for anesthesia-treated rats (35%) compared to naïve (28%). Kaplan-Meier survival analysis revealed a significant difference in the mortality rate of anesthesia-treated aged rats compared to naïve (p=0.010). In contrast to these findings, one study concluded that life expectancy was not reduced in aged Fischer 344 rats exposed to isoflurane anesthesia (Culley et al., 2006). The discrepancy between this study and my findings may be due to methodological differences, including: different ages of aged rats (18- versus 22-month-old), isoflurane exposure (1.8% for 4 hours versus 1.2% for 2 hours), and the length of time that survival was monitored post-anesthesia (3 months versus 10 months). Additionally, Culley and
colleagues (2006) did not separate aged animals based on spatial learning impairment, which may have concealed differences between groups. It has also been demonstrated that delirium and dementia are correlated to increased morbidity and mortality in elderly hospital patients (Cole and Primeau, 1993; Francis and Kapoor, 1992; Levkoff et al., 1991; Pompei et al., 1994; Rockwood et al., 1999). Therefore, it is possible that the observed cognitive deficits following anesthesia exposure in aged rats and humans may contribute to the increased mortality rates of the afflicted individuals.

**Proposed mechanisms of anesthesia-induced spatial learning deficits in aged rats**

The cellular mechanisms underlying anesthesia-induced cognitive deficits are likely multifactorial. Two separate mechanisms will be discussed in the next sections. The first involves a mechanism of isoflurane-induced cell death that has been suggested by studies of isoflurane-induced neurotoxicity *in vitro*. The second involves disruption of NMDA receptor mediated signaling pathways in the hippocampus of aged rats following isoflurane exposure *in vivo*. The latter is the proposed mechanism of spatial learning impairment in aged rats exposed to isoflurane anesthesia put forth by this dissertation.

**Cell death**

In Chapter III, isoflurane induced caspase-3 activation (Fig. 3.1) and cell death (Fig. 3.2) were demonstrated. These finding are supported in the literature. Various researchers have shown the inherent neurotoxicity of isoflurane anesthesia in culture (Head *et al*., 2009; Wang *et al*., 2008; Wei *et al*., 2008; Zhen *et al*., 2009). Additionally, reports implicate caspase-3 activation in isoflurane-induced apoptosis (Head *et al*., 2009; Jevtovic-Todorovic *et al*., 2003; Sanders *et al*., 2009; Wise-Faberowski *et al*., 2006).
Since caspase-3 activation represents one of the final steps in the apoptotic pathway, it is possible that various upstream effectors may be involved in isoflurane induced cell death.

Recent evidence suggests that the mitochondrial apoptotic pathway was activated by isoflurane via an increase in protein and mRNA levels of the pro-apoptotic factor bax, and a decrease in the anti-apoptotic factor bcl-2, ultimately leading to activation of caspase 3 and apoptosis in vitro (Zhang et al., 2010). Analysis of immunoblots of bax and bcl-2 from hippocampal and cortical cultures demonstrated no significant change in protein expression levels 30 minutes, 1 hour or 2 hours after isoflurane (Appendix A Figure 6.4). There is also the possibility caspase-3 activation is mediated by caspase 8 and/or caspase 9 via a path separate from bax and bcl-2, such as activation of death receptors at the cell surface or non-specific cellular insults. Another explanation is that isoflurane exposure may result in over stimulation of NMDA receptors, which has previously been shown to lead to caspase-3 activation and apoptotic cell death (Laabich and Cooper, 2000; Laabich et al., 2000; 2001; Lin et al., 2005). Since caspase-3 amplifies the apoptotic pathway at a late stage leading to cell death (reviewed Slee et al., 1999), treatment with isoflurane may represent a non-specific cellular insult that activates apoptosis and cell death. However, this may not represent an exclusive mechanism of cell damage in anesthesia treatment.

The anesthetic action of isoflurane mediates various neurotransmitter systems, including inhibition of neurotransmission via competitive antagonism of NMDA receptors (Hoffman et al., 1992; Franks and Lieb, 1994). In Chapter III, significant increases in protein expression of NMDA receptor subunits NR1 and NR2B were detected in isoflurane-treated neurons (Fig.3.1). Chronic blockade or stimulation of
NMDA receptors results in upregulation of the receptor (Gunduz-Bruce, 2009), providing further evidence for the potential over-stimulation of NMDA receptors by isoflurane. Additionally, treatment with Ro 25-6981, an NR2B specific NMDA receptor antagonist attenuated cell death in neuronal cultures (Fig. 3.2). These findings are similar to published reports that Memantine, an NMDA receptor antagonist, reduced isoflurane-induced caspase-3 activation (Zhang et al., 2008).

Isoflurane treatment induced cell death in neuronal cultures (Chapter III) similar to published reports (Head et al., 2009; Jevtovic-Todorovic et al., 2003; Lemkull et al., 2011; Sanders et al., 2009; Wang et al., 2008; Wei et al., 2008; Wise-Faberowski et al., 2006; Zhang et al., 2009; Zhen et al., 2009). However, in Chapter IV, cell counts performed in the hippocampus of aged rats exposed to isoflurane in vivo showed no cell loss at 3 months post-anesthesia (Figure 4.4) indicating minimal isoflurane-induced cell death in vivo. It is possible that some cell death occurs in the brains of aged rats at earlier time points following anesthesia. Additionally, more sensitive detection methods may be required to determine the extent of cell loss following anesthetic exposure in the aged brain.

In Chapter IV, aged animals exposed to isoflurane anesthesia in vivo did not show any change in caspase-3 activation. However, an age-dependent increase in caspase-3 activation was detected. Specifically, protein expression of the cleaved caspase-3 fragment (~17kD) was elevated in hippocampal and cortical immunoblots of 21-month-old animals compared to 18-month-old animals, regardless of anesthesia treatment in unimpaired (Fig. 4.1A-B) and impaired (Fig. 4.8A-B) aged rats. Age-related caspase-3 activation has been previously demonstrated in the literature. Evidence from
immunoblotting and immunostaining studies revealed caspase-3 activity is significantly increased in the hippocampus of aged (22-month-old) compared to young (4-month-old) Wistar rats (Lynch and Lynch, 2002). Since caspase-3 is a reliable marker of apoptotic cell death (Green and Reed, 1998; Kuan et al., 1999), it is possible that the aged brain has an inherent program of increased susceptibility to activation of caspase-3 mediated cell death. Although pro-caspase-3 levels are not elevated in the macaque brain, altered cellular distribution is evident in the hippocampus (Zhang et al., 2006), further suggesting increased vulnerability to apoptotic stimuli in the aged brain.

It is also possible that the activation of caspase-3 in the aged brain may be involved in non-apoptotic functions recently identified in neuronal cells, particularly in synaptic plasticity and learning and memory processes (D’Amelio et al., 2010). It is important to note that various capase-3 substrates are linked to neuronal plasticity (Chan et al., 1999). Therefore, age-related changes in caspase-3 activity would have downstream effects on learning and memory targets in the aged brain. Furthermore, caspase-3 is reported to be involved in molecular mechanisms underlying learning and memory in various species, such as long-term plasticity in the snail (Bravarenko et al., 2006), long-term habituation to a song in the zebra finch (Huesmann and Clayton, 2006), and LTP in the rat hippocampus (Gulyaeva et al., 2003). Inhibition of caspase-3 in the rat brain reduced avoidance learning (Stepanichev et al., 2005), further indicating a role for caspase-3 activity in learning and memory processes. Therefore, age-related changes in the activity of caspase-3 may contribute to spatial learning deficits observed in aged individuals. Anesthesia may interact with caspase-3 associated changes in learning and memory mechanisms further explaining anesthesia-related spatial learning impairments.
Additionally, the discrepancy between isoflurane-induced caspase-3 activation in studies of neuronal cultures and anesthetic treatment in aged rats may be due to inherent differences between studies performed \textit{in vitro} and \textit{in vivo}. Neuronal cell cultures were harvested from developing rat brains, which are known to be highly sensitive to the effects of anesthetics (Ikonomidou \textit{et al}., 1999; 2000; Jevtovic-Todorovic \textit{et al}., 2003; Loepke \textit{et al}., 2006; Yon \textit{et al}., 2005; 2006). Additionally, studies performed \textit{in vitro}, where caspase-3 activation was monitored from 30 minutes to 2 hours after isoflurane exposure, were unable to mimic the anesthetic conditions and effects in the CNS during anesthetic exposure. In an organism, such as a rodent or human exposed to anesthesia, the brain may not endure the same intensity of cellular injury compared to anesthetic exposure in culture. In an organism, the amount of isoflurane that reaches the brain is measured by the partial pressure of isoflurane in arterial blood, which is 50-60\% of the concentration in humans (Kennedy and Longnecker, 1996) and rats (Holdcroft \textit{et al}., 1999). During isoflurane anesthesia treatment \textit{in vivo}, neurons are exposed to less of the anesthetic agent than neurons in culture, which may explain the conflicting findings between the studies. Taken together, it is clear that isoflurane anesthetic exposure results in damage to neurons in culture via capase-3 activation. However, this does not represent an exclusive mechanism of damage to neurons by isoflurane.

\textbf{NMDA receptor subunit NR2B}

Several studies have suggested a change in the role of NMDA receptors in the aged brain. High density NMDA receptor binding in the hippocampus of aged rats is associated with poor long-term memory retention in the Morris water maze (Topic \textit{et al}., 2007). Unimpaired aged rats, tested in a spatial learning paradigm, exhibited higher age-
related declines in binding of MK801 in the hippocampus and cortex compared to impaired aged rats (Le Jeune et al., 1996). Additionally, non-NMDA receptor-dependent LTP and long term depression (LTD) are associated with better memory performance in aged rats than NMDA-mediated synaptic plasticity (Boric et al., 2008; Lee et al., 2005). Also, the NMDA receptor antagonist, Memantine, improved memory performance (Beracochea et al., 2008; Danysz and Parsons, 2003; Norris and Foster, 1999) and increased neurogenesis (Nacher et al., 2003) in aged subjects. These studies provide evidence suggesting that a functional change in the NMDA receptor occurs in the aged brain.

In Chapter IV, mechanisms underlying isoflurane-induced spatial learning deficits, specifically involving NMDA receptor protein expression changes in the aged rat brain were investigated. Normal aging brings about a decrease in binding strength and a decline in NR2B subunit protein expression (reviewed Magnusson et al., 2010). Therefore, age-related alterations in protein and mRNA expression of NMDA receptor subunits in the aged brain may lead to a functional change in the remaining NR2B-containing NMDA receptors. In aged rodents, individuals with high levels of NR2B protein expression demonstrate the poorest spatial learning performance, whereas young animals with high levels of NR2B subunit protein expression in the hippocampus demonstrate improvement in spatial learning performance (Rammes et al., 2009; Zhao et al., 2009).

The functional switch in the NR2B-containing NMDA receptors during aging may be explained by a shift in protein expression with more extra-synaptic localization of NR2B. Such a shift has been shown to increase LTD (Massey et al., 2004) and increase
activation of a CREB (cAMP response element binding) shut-off mechanism, interfering with brain derived neurotrophic factor (BDNF) levels, loss of mitochondrial membrane potential, and cell death (Hardingham et al., 2002). However, we were unable to detect anesthesia-induced cell loss in aged rats 3 months post-anesthesia (Figure 4.4).

Because isoflurane modulates NMDA receptors (Hoffman et al., 1992), it is likely that prolonged isoflurane exposure induces a change in protein expression of NMDA receptor subunits. Based on the idea that a functional switch occurs in NR2B-containing NMDA receptors such that high levels correspond to poor spatial learning performance, I hypothesized that isoflurane anesthesia induces an increase in NMDA receptor NR2B subunit protein expression in the aged rat hippocampus leading to anesthesia induced spatial learning impairments in aged rats.

In Chapter IV, aged rats exposed to isoflurane anesthesia displayed higher levels of NR2B protein expression in the hippocampus and cortex immediately following anesthetic exposure. The elevated NR2B levels were maintained up to 3 months post-anesthesia. The isoflurane-induced elevation in NR2B protein expression is supported in the literature. In one study male C57BL6/J adult (4-5 months) mice were exposed to isoflurane anesthesia for 2 hours. Learning and memory, LTP in vitro, and NR2B protein expression were evaluated at 24 hours post-anesthesia. Protein expression analysis revealed an increase of NR2B in hippocampal neurons after anesthesia (Rammes et al., 2009). In contrast, Rammes and colleagues (2009) demonstrated improvements in spatial learning performance in young rats following isoflurane anesthetic exposure. The discrepancy in behavioral outcomes following isoflurane most likely involves the age of the rodents at the time of anesthetic exposure.
Aged rats treated with anesthesia, demonstrate chronic spatial learning impairments at 3 months post-anesthesia (Figures 4.2 and 4.3). Previous reports demonstrating spatial learning deficits that correspond to high levels of NR2B in aged rodents (Zhao et al., 2009) support the finding that NR2B protein expression levels are chronically elevated in the brains of aged rats that exhibit anesthesia-induced spatial learning deficits. Taken together, these findings suggest that a functional switch occurs for NR2B in the brain between young adult and aged animals and isoflurane-induced increases in NR2B result in poor spatial learning outcomes for aged animals. The isoflurane-induced change in NR2B protein expression in the aged hippocampus likely contributes to disruption in downstream signaling molecules, such as ERK.

pERK

NMDA receptor subunit composition and localization has specific consequences for activation of ERK. NR2A-containing NMDA receptors are coupled to the activation of ERK, while NR2B-containing NMDA receptors are associated with ERK inhibition (Kim et al., 2005). Additionally, the location of NMDA receptors, whether synaptic or extrasynaptic, can have differential effects on ERK activation (Hardingham, 2006). See Figure 1.3 for an illustration of the opposite effects on ERK1/2 when synaptic versus extrasynaptic NMDA receptors are activated. Therefore, it is possible that an increase in synaptic NR2A-containing NMDA receptors improves spatial learning performance, while elevation of extrasynaptic NR2B-containing NMDA receptors inhibits spatial learning. I hypothesized that elevated levels of NR2B result in a disruption in downstream signaling cascades, specifically in ERK1/2 activation in the hippocampus and cortex of aged rats following isoflurane anesthesia exposure.
In Chapter IV anesthesia treated rats displayed a decrease in phospho-ERK1/2 (pERK), a measure of ERK activation, at 3 months post-anesthesia. Because hippocampal ERK activation is required for spatial learning (Atkins et al., 1998; Selcher et al., 1999), a reduction in ERK activation would result in poor spatial learning performance in these animals. Hippocampal pERK levels were significantly reduced at 3 months, but not at 2 weeks. This finding is consistent with the appearance of spatial learning deficits in anesthesia-treated aged rats at 3 months, but not at 2 weeks. It is possible that the acute elevation in NR2B may have little impact on pERK levels in the hippocampus. However, prolonged, chronic elevation in NR2B protein expression in the aged rat brain leads to a disruption in ERK activation over time. The minimal disturbance in ERK activation, undetectable at early time points, may not have an immediate behavioral consequence. However, the accumulative reduction in activated ERK, apparent at later time points, results in chronic spatial learning impairments in aged rats.

ERK can be activated by various mechanisms including but not limited to NMDA receptors. Once activated ERK exerts many downstream effects, including: the regulation of cellular excitability, activation of transcription factors leading to protein synthesis, changes in gene expression, dendritic spine stabilization, modulation of ion channels, and receptor insertion (reviewed in Sweatt, 2004). All cellular events can ultimately influence synaptic plasticity in the hippocampus with cognitive effects. Dysfunction in ERK signaling can have devastating consequences for learning and memory processes. A group of researchers have linked dysfunction in the ERK pathway to neurofibromatosis type 1 (NF1)-associated mental retardation (Costa et al., 2001; 2002; Ohno et al., 2001; Silva et al., 1997). Others have demonstrated that inhibition of ERK disrupts regulation
of membrane electrical properties that control back-propagating action potentials and NMDA receptor activation (Watanabe et al., 2002; Winder et al., 1999). ERK has also been implicated in formation and stabilization of dendritic spines in hippocampal neurons (Goldin et al., 2003; Wu et al., 2001) and local dendritic protein synthesis (Kelleher et al., 2004). Additionally, recent evidence suggests that ERK may be involved with signal integration over long periods of time (Wu et al., 2001). The far reaching effects of ERK and ERK disruption in the hippocampus suggest that interference in pERK signaling by isoflurane anesthesia in the aged brain can have catastrophic effects on hippocampal dependent learning and memory processes. Taken together, isoflurane-induced chronic reduction in activated ERK in the hippocampus of aged rats at later time points may produce a number of downstream effects involved in synaptic plasticity, LTP, and learning processes in the hippocampus, resulting in the long term impairment in spatial learning demonstrated in anesthetized rats.

**Proposed mechanism**

Taken together, the findings from my investigation of anesthesia-induced spatial learning impairment in aged rats suggest the following sequence of events. During normal aging, a functional switch in NR2B-containing NMDA receptors occurs, such that high levels of the protein correspond to poor spatial learning outcomes in aged rats. Isoflurane exposure augments this effect, leading to an acute increase in NR2B protein expression in the hippocampus and cortex of aged rats. The elevated levels of NR2B are sustained and present at chronic time points up to 3 months post-anesthesia. The prolonged elevation of NR2B protein expression leads to dysfunction in downstream signaling kinases, ERK1/2. The reduction in phospho-ERK, the activated form of the
kinase, contributes to chronic spatial learning impairment in aged rats exposed to isoflurane anesthesia. The proposed mechanism of isoflurane induced spatial learning deficits in aged rats is presented in the schematic below (in the aged brain):

Isoflurane exposure → Acute increase in NR2B → Chronic increase in NR2B → decrease in pERK → spatial learning impairment in anesthesia-treated aged rats

Figure 5.2 conceptualizes the proposed mechanism of isoflurane anesthesia-induced learning impairment in hippocampal neurons of the aged rat brain. Briefly, NMDA receptors containing NR2A and NR2B subunits are found at synaptic and extrasynaptic locations on the postsynaptic cell. NR2A-containing NMDA receptors are mainly localized to synaptic locations, while NR2B-containing NMDA receptors are primarily found extrasynaptically (Liu et al., 2004; Stocca and Vicini, 1998; Tovar and Westbrook, 1999). Release of glutamate binds NMDA receptors, and simultaneous depolarization results in an influx of calcium ions. The calcium influx leads to activation or inhibition of protein kinases (CaMK’s and ERK), depending on the localization of the NMDA receptors. Specifically, extrasynaptic NMDA receptors inhibit the downstream kinases, whereas synaptic NMDA receptors activate the kinases. (1) During isoflurane exposure, isoflurane, an NMDA receptor antagonist, blocks NMDA receptors. (2) The chronic antagonism of NMDA receptors leads to an upregulation of receptors. (3) This elevation leads to an increase in inhibition of downstream signaling molecules. Reduction in ERK activation contributes to the observed anesthesia-induced spatial learning deficits in aged rats.
Pharmacological treatments

Inflammatory cytokines, IL-1β and IL-18, contribute to neuropathology of age-related diseases such as Alzheimer’s and Parkinson’s disease (Chiarini et al., 2006; Di Bona et al., 2008; Tansey et al., 2007) and normal cognitive decline associated with aging (Ershler et al., 1993; Lee et al., Lynch, 2002; 2000; Pugh et al., 2001; Rubenoff et al., 1998; Wilson et al., 2002; Yirmiya et al., 2002). Due to the positive feedback of these cytokines on inflammatory processes, it is possible that therapeutic drugs aimed at inhibition of inflammasome activation may provide anti-inflammatory treatments that improve cognitive outcomes for elderly individuals. Additionally, the success of probenecid treatment in improving spatial learning performance in aged rats presented in Chapter II, suggests that anti-inflammasome treatments may offer additional treatment options for cognitive decline associated with normal aging and/or neurodegenerative diseases to researchers and clinicians in the future.

Therapeutic treatments for POCD are of great significance to geriatric anesthesiologists and elderly surgical patients. However, currently no pharmacological treatment for POCD exists. The current findings have implications for a mechanism of isoflurane-induced increase in NR2B hippocampal protein levels in aged rats, while also suggesting potential therapeutic targets for treatment of POCD. In Chapter IV, Ro 25-6981 (Ro), the NR2B specific blocker, attenuated an elevation in NR2B protein expression in the hippocampus. This NR2B elevation is suggested to trigger dysfunction in downstream ERK activation, eventually leading to spatial learning impairments in aged rats. The success of Ro pretreatment in the aged brain suggests a role for NMDA receptor antagonists in the potential prevention of anesthesia induced cognitive deficits.
To date, it has not been approved for use in humans. However, NMDA receptor antagonists are currently available for off label treatment. One potential therapeutic approach to POCD suggested by the present observations would be the preoperative administration of an NMDA receptor antagonist. Memantine, a noncompetitive partial antagonist of the NMDA receptor, is one such possibility. Memantine is utilized in the treatment of moderate to severe AD (Mount and Downton, 2006) and has been used in anesthesia for the treatment of neuropathic pain (Buvanendran and Kroin, 2008). Memantine may be protective under anesthetic conditions and is therefore of potential interest in the prevention of POCD. Further research involving animal and clinical studies is necessary to determine the mechanisms for POCD and the potential therapeutic effects of an NMDA receptor antagonist, like Memantine, on the treatment and prevention of POCD.

**Future experiments**

My studies have opened various lines of possible research topics. First, since the NLRP1 inflammasome is implicated in age-related cognitive decline, it is possible that the inflammasome may contribute to pathological inflammatory processes related to age-related neurological disorders. Second, investigation of the effects of isoflurane on LTP and other mechanisms of synaptic function in the hippocampus would provide additional support for the findings presented here, clarifying the underlying mechanisms of isoflurane-induced spatial learning impairment. Third, since my studies have indicated elevated activation of NLRP1 inflammasome in the aged brain, a logical next step would be to investigate the effects of isoflurane on inflammasome activation.
Although NR2B-mediated reduction in ERK activation is presented here, downstream effects of ERK disruption could be investigation in the hippocampus following anesthetic exposure. Studies of LTP disruption performed in hippocampal slice preparations may provide evidence that supports a disruption in LTP, dendritic spine protein synthesis, gene expression changes, and a number of other downstream targets implicated in ERK activation. Further study is required to pinpoint synaptic plasticity mechanisms that are disrupted by isoflurane anesthesia exposure in the aged brain. It is also possible that the aged brain has a greater vulnerability in the previously mentioned mechanisms that increases the susceptibility of the anesthetic insult leading to cognitive deficits.

The involvement of NLRP1 inflammasome activation in age-related neurodegenerative diseases represents a future avenue of research. It is possible that the NLRP1 inflammasome plays a role in neuropathological conditions such as AD. IL-1β has been implicated in AD pathology. IL-1 has been shown to stimulate β-amyloid precursor protein (APP) processing and synthesis (Del Bo et al., 1995; Grilli et al., 1996; Goldgaber et al., 1989; Yang et al., 1998) and Aβ production (Blasko et al., 1999), indicating a role for IL-1 in Aβ fibrillisation and aggregation, key events in AD (Shastry, 2003). Additionally, AD is associated with increased expression of caspase-1 (Zhu et al., 1999) in the brain. Therefore, NLRP1 inflammasome activity, which involves caspase-1 activation and IL-1β maturation and secretion, may be involved in AD pathology. Furthermore, inhibition of NLRP1 inflammasome activity, with a drug such as probenecid, may reduce some of the physiological effects associated with AD.
The interaction of age and anesthesia presents a complicated area of study. The findings presented here implicate a role for inflammation via NLRP1 inflammasome activation in age-related spatial learning impairment. It would be interesting to determine whether inflammasome activation is exacerbated by isoflurane anesthesia exposure in the aged brain, contributing to anesthesia induced spatial learning deficits. It would take careful interpretation to determine cause and effect relationships of the role of inflammasome activation in the anesthesia treated aged brain. However, it would provide valuable information for anesthesiologists. Additionally, general anesthetic exposure is required for major surgery, which presents another potential initiator of inflammatory processes in the CNS. Therefore, careful dissection of the roles of surgery versus anesthesia is necessary to determine individual contributions to cognitive outcomes. It is also possible that there is an additive effect of surgery and anesthesia exposure, such that the whole effect is greater than the sum of the individual parts. Future experiments aimed at determining the effects of the combination of surgery and anesthesia on cognitive outcomes in elderly patients would further our understanding of POCD.

Taken together, my studies have provided evidence for NLRP1 inflammasome activity increases in the aged brain, and suggested a novel mechanism of anesthesia-induced spatial learning deficits in aged rats involving the NMDA receptor subunit NR2B. Various therapeutic targets for improvement in cognitive impairment in the aged brain have been identified in the course of these investigations. Finally, these studies have opened several avenues for future lines of research in the fields of aging, anesthesia, and inflammation benefitting basic science researchers and clinicians alike.
### Tables for Chapter V

<table>
<thead>
<tr>
<th>NLRP1 Inflammasome Component</th>
<th>Function</th>
<th>Normal aging</th>
<th>Probenecid treatment</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>Pro-inflammatory cytokine</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>IL-18</td>
<td>Pro-inflammatory cytokine</td>
<td>↑</td>
<td>Highly variable between individuals</td>
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<tr>
<td>Caspase-1</td>
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<td>↑ (2 fold)</td>
<td>↓</td>
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<tr>
<td>Caspase-11</td>
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<td>↑ (1.5 fold)</td>
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<td>No change</td>
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<td>Adaptor protein</td>
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<tr>
<td>PanX1</td>
<td>Pore forming protein</td>
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<td>↓</td>
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**Table 5.1** Summary of findings for the NLRP1 inflammasome activation in the aged rat brain before and after probenecid treatment.

<table>
<thead>
<tr>
<th>Isoflurane exposure induces:</th>
<th>In vitro</th>
<th>Cell type</th>
<th>In vivo</th>
<th>Brain region</th>
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<tr>
<td>Increase in Bax (pro-apoptotic)</td>
<td>YES&lt;sup&gt;1-5&lt;/sup&gt;</td>
<td>Rat PC12 pheochromocytoma cells and primary cortical neurons&lt;sup&gt;1&lt;/sup&gt;, H4-APP&lt;sup&gt;2-5&lt;/sup&gt;</td>
<td>NO&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Hippocampus and cortex</td>
</tr>
<tr>
<td>Decrease in Bcl-2 (anti-apoptotic)</td>
<td>YES&lt;sup&gt;1-5&lt;/sup&gt;</td>
<td>Rat PC12 pheochromocytoma cells and primary cortical neurons&lt;sup&gt;1&lt;/sup&gt;, H4-APP&lt;sup&gt;2-5&lt;/sup&gt;</td>
<td>NO&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Hippocampus and cortex</td>
</tr>
<tr>
<td>Caspase-3 activation</td>
<td>YES&lt;sup&gt;1-7&lt;/sup&gt;</td>
<td>H4-APP&lt;sup&gt;2-5&lt;/sup&gt;, CD3 T lymphocytes and Jurkat T cells&lt;sup&gt;6&lt;/sup&gt;, chicken B-lymphocytes&lt;sup&gt;7&lt;/sup&gt;</td>
<td>NO&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Hippocampus and cortex</td>
</tr>
<tr>
<td>Increase in NR2B</td>
<td>YES&lt;sup&gt;8,9&lt;/sup&gt;</td>
<td>Hippocampal slice preparation&lt;sup&gt;8&lt;/sup&gt;, Hippocampal and cortical primary neurons</td>
<td>YES&lt;sup&gt;8,11&lt;/sup&gt;</td>
<td>Hippocampus and cortex</td>
</tr>
<tr>
<td>Increase in NR1</td>
<td>YES&lt;sup&gt;8,9&lt;/sup&gt;</td>
<td>Hippocampal slice preparation&lt;sup&gt;8&lt;/sup&gt;, Hippocampal and cortical primary neurons</td>
<td>YES&lt;sup&gt;8,11&lt;/sup&gt;</td>
<td>Hippocampus and cortex</td>
</tr>
</tbody>
</table>

<sup>1</sup>Wei et al., 2005; <sup>2</sup>Zhang et al., 2010; <sup>3</sup>Xie et al., 2006; <sup>4</sup>Xie et al., 2007; <sup>5</sup>Xie et al., 2008; <sup>6</sup>Loop et al., 2005; <sup>7</sup>Wei et al., 2008; <sup>8</sup>Rammes et al., 2009; <sup>9</sup>Figure 3.1; <sup>10</sup>Appendix A Figure 6.4; <sup>11</sup>Figure 4.3

**Table 5.2** Comparison of isoflurane-induced changes in protein expression of Bax, Bcl-2, Caspase-3 cleaved fragment, NR2B, and NR1 between *in vitro* and *in vivo* studies.
Figures for Chapter V

Figure 5.1 Proposed mechanism of interaction between IL-1β and NMDA receptors in age-related cognitive decline.

Activation of the inflammasome complex results in maturation and secretion of the pro-inflammatory cytokine, IL-1β, contributing to elevated levels of the cytokine in the aged brain. Colocalization of IL-1 receptor (IL-1R) and N-methyl-D-aspartate (NMDA) receptors facilitate the phosphorylation of NMDA receptors by IL-1R activation, which may lead to over-stimulation of NMDA receptor by agonists, such as Glutamate (Glut), released from the presynaptic cell. Over-stimulation of extrasynaptic NMDA receptors can lead to impairment in LTP and learning and memory processes, and caspase-3 mediated cell death.
Figure 5.2. Proposed mechanism of isoflurane induced spatial learning impairment in the aged brain.

Before isoflurane anesthesia exposure, glutamate binds to NMDA receptors on the postsynaptic cell. Upon depolarization, NMDA receptor channels open allowing a calcium (Ca2+) influx. Depending on localization and subunit composition downstream kinases (CaMKI, CaMKII, ERK) are either activated or inhibited. Synaptic, NR2A-containing NMDA receptor activation stimulates activation of the kinases. NR2B-containing NMDA receptors inhibit downstream kinases. During isoflurane exposure, (1) isoflurane, a competitive NMDA receptor antagonist, binds NMDA receptors, regardless of subunit composition and localization. Isoflurane blocks the receptor for the duration of exposure. Chronic antagonism of NMDA receptors by isoflurane results in an (2) upregulation of NR2B-containing NMDA receptors in the hippocampus and cortex of aged rats long after the anesthetic has cleared. After isoflurane exposure, the increase in NR2B containing NMDA receptors results in a higher ratio of NR2B to NR2A, and consequently, (3) greater inhibition of downstream learning and memory signaling kinases such as ERK, CREB, and CaMKII. Thus, learning and memory impairments are observed in these animals at chronic time points.
Figure 6.1 Distribution of spatial learning scores for young and aged rats.
Young (3 month old, white bars) and aged (18 month old, black bars) were submitted to a 3-day spatial acquisition task via Morris water maze, with an average latency to platform measure taken on day 3. The number of animals in each range of scores is plotted. Young animals demonstrate a standard distribution of scores. Aged animals demonstrated a bimodal distribution of scores.
Figure 6.2 Spatial learning performance of young and aged-impaired and aged-unimpaired rats before anesthetic exposure.

Young (3 month old, open circles, dotted line) and aged (18 month old) –impaired (open squares, dashed line) and –unimpaired (closed triangles, solid line) rats were tested in a spatial acquisition task via Morris water maze. Aged rats were separated into impaired and unimpaired groups based on day 3 average latency scores, such that impaired rats had an average latency >30s on day 3, whereas unimpaired rats had an average latency <30s. (A) Latency to platform and (B) mean path length are plotted. [n=15-17/group, means plotted, error bars represent SEM, *p<0.05 compared to young, **p<0.01 compared to young, # p<0.05 compared to unimpaired.]
Figure 6.3 Mortality and 3 month survival curves for aged rats treated with isoflurane anesthesia versus naïve.

(A) Mortality rate, designated by percent death at 3 months post-anesthesia treatment, for aged (impaired and unimpaired) rats. Aged rats were 18 months old at time 0. Anesthesia exposure occurred just after time 0. Aged rats designated as impaired by behavioral baseline testing demonstrated a higher mortality rate for anesthesia treated (50.0%) than naïve (35.3%). For unimpaired rats, the mortality rate for anesthesia treated was higher (37.5%) versus naïve (28.6%). (B) Kaplan-Meier survival analysis by log ranks was performed on each group for the survival period three months post-anesthesia treatment. There was a statistically significant difference between survival curves (p=0.010) based on anesthetic treatment.
Figure 6.4 Immunoblot analysis of Bax and Bcl-2 in hippocampal and cortical neurons treated with isoflurane (Iso) in vitro.

Primary hippocampal and cortical neurons were treated with isoflurane (Iso) for 30m, 1h, 2h, or no isoflurane (Control). Quantification of immunoblots for (A) Bax and (B) Bcl-2 are plotted. No significant differences were detected between groups. [Data are presented as means; error bars represent +/- standard error mean (+/-SEM); n=5/group.]
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