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Isoflurane/nitrous oxide anesthesia induces increases in NMDA receptor subunit NR2B protein expression in the aged rat brain

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Abstract

Postoperative cognitive dysfunction, POCD, afflicts a large number of elderly surgical patients following surgery with general anesthesia. Mechanisms of POCD remain unclear. N-methyl-D-aspartate (NMDA) receptors, critical in learning and memory, that display protein expression changes with age are modulated by inhalation anesthetics. 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. Three-month-old and 18-month-old male Fischer 344 rats were randomly assigned to receive 1.8% isoflurane/70 % nitrous oxide (N₂O) anesthesia for 4h or no anesthesia. Spatial learning was assessed at 2 weeks and 3 months post-anesthesia in Morris water maze. Hippocampal and cortical protein lysates of 18-month-old rats were immunoblotted for activated caspase 3, NMDA receptor subunits, and extracellular-signal regulated kinase (ERK) 1/2. In a separate experiment, Ro 25-6981 (0.5mg/kg dose) was administered by I.P. injection before anesthesia to 18-month-old rats. Immunoblotting of NR2B was performed on hippocampal protein lysates. At 3 months post-anesthesia, rats treated with anesthesia at 18-months-old demonstrated spatial learning impairment corresponding to acute and long-term increases in NR2B protein expression and a reduction in phospho-ERK1/2 in the hippocampus and cortex. Ro 25-6981 pretreatment attenuated the increase in acute NR2B protein expression. Our findings suggest a role for disruption of NMDA receptor mediated signaling pathways in the hippocampus and cortex of rats treated with isoflurane/ N₂O anesthesia at 18-months-old, leading to spatial learning deficits in these animals. A potential therapeutic intervention for anesthesia associated cognitive deficits is discussed.

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Keywords

aging; anesthesia; extracellular signal regulated kinase (ERK); isoflurane; N-methyl-D-aspartate (NMDA) receptor subunit NR2B; Ro 25-6981

1. Introduction

POCD is described as cognitive deficits in memory and concentration following surgery and exposure to general anesthesia. Advanced age is the primary risk factor for development of POCD (Moller et al., 1998; Monk et al., 2008). Accordingly, POCD is observed in 10-25% of elderly patients (Fong et al., 2006; Newman et al., 2007). The phenomenon of age-dependent differences in response to anesthesia occurs both clinically and in animal models. It is known that anesthetics alter learning and memory performance in rodents. However, reports are inconsistent, and highly dependent on the age of the animal at the time of the exposure. Some investigators report cognitive improvements following anesthetic exposure in young rodents (Komatsu et al., 1993; Culley et al., 2003; Rammes et al., 2009), whereas others describe anesthesia-induced cognitive impairments in aged, 18-month-old rats (Culley et al., 2003; 2004). However, a neurobiological mechanism for the interaction of anesthesia and the aged brain resulting in lasting cognitive deficits remains lacking in the literature, despite its prevalence in humans and rodents.

Inhalation anesthetics, including isoflurane, modulate NMDA-type glutamate receptors to produce analgesic and anesthetic actions in the central nervous system (CNS) (Hoffman et al., 1992; Yamakura and Harris, 2000; Raines et al., 2001; Hara et al., 2002; Eger et al., 2006; Solt et al., 2006). Due to the critical role of NMDA receptors in learning and memory processes (reviewed Morgado-Bernal, 2011); these receptors may play a role in anesthesia-induced cognitive deficits in the aged brain. Moreover, alterations in protein and mRNA expression occur in the aged brain (Magnusson et al., 2010). It has been suggested that these age-related changes may involve a functional change in NMDA receptor function such that high levels of NMDA receptor subunits NR1 and NR2B protein expression correspond to improved spatial learning performance in 4-5 month-old mice (Rammes et al., 2009; Zhao et al., 2009) and poor outcomes in 26-month-old mice (Zhao et al., 2009). Based on the known properties of NMDA receptors, we hypothesized that anesthesia-induced spatial learning deficits in aged rats may involve long-term disruption of NMDA receptor-mediated learning and memory signaling pathways. Here, we investigated the effects of isoflurane/ N₂O anesthesia, a commonly used anesthetic combination, on protein expression levels of NMDA receptor subunits and downstream signaling molecules, extracellular-signal regulated kinases (ERK) in the hippocampus and cortex of 18-month-old Fischer 344 rats. Our findings corroborate previous studies describing anesthesia-induced spatial learning deficits in aged rats (Culley et al., 2003; 2004; 2007) and implicate the involvement of the NMDA receptor subunit NR2B in an underlying mechanism of delayed cognitive impairments in the aged brain following general anesthesia *in vivo*.

2. Results

2.1 Isoflurane/ N₂O anesthesia induced spatial learning deficits in 18 month old Fischer 344 rats at 3 months post-anesthesia

Behavioral testing was performed at three separate time points, a baseline performance one week before anesthetic exposure (days -7 to -5), and 2 weeks (days 14 to 16) and three months post-anesthesia (days 90 to 92) (N=12-15/group). Three-month-old rats treated with anesthesia demonstrated no significant differences from naïve age-matched counterparts at any of the three time points tested in the Morris water maze (data not shown). The

behavioral results for 18-month-old rats are shown in Figure 1. Rats treated with anesthesia at 18-months-old (N=15) displayed significantly higher latencies ($p=0.0043$, Fig. 1A) and mean path length ($p=0.003$, Fig. 1B) versus naïve age-matched rats (N=12) at 3 months post-anesthesia (day 92). Additionally, anesthesia treated 18-month-old rats demonstrated higher mean path length at 3 months post-anesthesia compared to baseline ($p<0.001$, Fig. 1B). From day 17 to 92 the mean path length increased significantly ($p=0.013$, Fig. 1B) for anesthesia treated 18-month-old rats compared to naïve age-matched controls. Swim speeds were similar between groups (data not shown). Overall, we showed that 18-month-old rats exposed to isoflurane/ N_2O anesthesia suffered deficits in spatial learning acquisition observable at 3 months post-anesthesia.

2.2 Isoflurane/ N_2O anesthesia had no effect on caspase 3 activation in the hippocampus and cortex of 18 month old Fischer 344 rats *in vivo*

One hypothesis regarding anesthetic neurotoxicity involves caspase 3 activation and inflammatory processes in the CNS (Xie et al., 2006; 2008, Zhang et al., 2008). To determine caspase 3 activation levels of 18-month-old Fischer 344 following isoflurane/ N_2O anesthetic exposure, animals were terminated by decapitation immediately, 2 weeks and 3 months post-anesthesia (N=5-6/group) and compared to naïve rats at 18-months-old that were terminated by decapitation immediately or 3 months later. Hippocampal and cortical tissues from experimental groups (see supporting material Fig. S1 for diagram of experimental groups) were subjected to western blot analysis with antibodies against caspase 3. Representative blots for hippocampus and cortex are shown (Fig. 2). In the hippocampus, caspase 3 activation, measured by caspase 3 fragment (17 kD) quantification, was elevated in naïve rats and anesthesia treated rats at 21 months compared to 18-month-old naïve rats ($p=0.0036$, $p=0.0031$, respectively, Figure 2A). Similarly in the cortex, naïve and anesthesia-treated rats demonstrated higher levels of caspase 3 fragment than naïve 18 months rats ($p=0.042$, $p=0.0039$, respectively, Figure 2B).

2.3 Effect of isoflurane/ N_2O anesthesia on protein expression levels of NMDA receptor subunits in the 18-month-old rat brain

In Figure 3, hippocampal and cortical brain samples from 18-month-old rats from experimental conditions (see supporting material Fig. S1 for diagram of experimental groups) (N=5-6/group) 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. In the hippocampus, no differences were detected between anesthesia and naïve groups for NR2A subunit. However, an age-related increase in NR2A was detected in both groups at 21 months compared to 18-month naïve rats ($p<0.001$, Fig. 3A). Rats treated with anesthesia at 18-months demonstrated an acute increase in NR2B compared to naïve in the hippocampus ($p=0.008$, Fig. 3A) and the cortex ($p=0.007$, Fig. 3B). A long-term increase in NR2B protein expression was also detected between anesthesia treated rats at 3 months compared to naïve 18-month rats (hippocampus: $p=0.002$; cortex: $p=0.003$) and compared to 21-month naïve rats (hippocampus: $p=0.006$; cortex: $p=0.006$). NR1 protein expression levels were elevated in anesthesia treated rats at 21 months compared to 18-month naïve rats in the hippocampus ($p=0.008$, Fig. 3A) and cortex ($p=0.007$, Fig. 3B). Additionally, NR1 protein levels were increased for 21 month versus 18-month-old naïve rats in the hippocampus ($p=0.002$, Fig. 3A).

2.4 Effect of isoflurane/ N_2O anesthesia on ERK1/2 activation in the brain of 18-month-old Fischer 344 rats

Activation of ERK1/2, a downstream kinase of NMDA receptors, is required for hippocampal dependent spatial learning (Atkins et al., 1998; Selcher et al., 1999).

Hippocampal and cortical brain samples all experimental conditions (see supporting material Fig. S1 for diagram of experimental groups) (N=5-6/group) were immunoblotted for phospho-ERK1/2 and total ERK1/2 to determine changes in activation of ERK1/2, the ratio of phospho- to total-ERK, following isoflurane/ N₂O anesthesia (Fig. 4). 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. 4A) and cortex (pERK1: p=0.002, pERK2: p=0.001, Fig. 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. 4B).

2.5 Ro 25-6981 treatment attenuated anesthesia-induced increase in NR2B subunit protein expression in the hippocampus of 18-month-old Fischer 344 rats immediately following isoflurane/ N₂O anesthesia

In Figure 5, Ro 25-6981 (Ro) pretreatment reduced hippocampal NR2B protein expression in 18-month-old rats exposed to isoflurane/ N₂O anesthesia treatment (N=6/group). Hippocampal NR2B protein levels were significantly reduced (P=0.008, Figure 5) 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/ N₂O were not significantly different from naïve 18-month-old rats.

3. Discussion

There is extensive literature reporting incidences of cognitive deficits following general anesthesia in aged humans (Moller et al., 1998; Johnson et al., 2002; Canet et al., 2003;; Newman et al., 2007; Monk et al., 2008; Deiner and Silverstein, 2009), and rodents (Culley et al., 2003, 2004, 2007). In clinical studies, the detection of POCD occurs within weeks following anesthetic exposure and manifests as long-term cognitive deficits on cognitive tasks (Moller et al., 1998; Biedler et al., 1999; Monk et al., 2008;). There may be an interaction of advancing age and anesthetic insult that result in cognitive dysfunction for some elderly patients. Here, we investigated spatial learning performance following anesthetic exposure at early (2 weeks) and late (3 months) time points post-anesthesia in 3-month-old and 18-month-old Fischer 344 rats. To identify protein expression changes in activated caspase 3 and NMDA receptor subunits, hippocampal and cortical protein lysates of brains from rats exposed to isoflurane/nitrous oxide anesthesia at 18-months-old were assayed by western blot analysis at various time points following anesthetic exposure. Our findings identify anesthesia-induced increases in hippocampal and cortical NMDA receptor subunit NR2B protein expression at early and late time points corresponding to poor behavioral performance in Fischer 344 rats exposed to isoflurane/nitrous oxide anesthesia at 18-months of age.

In a hippocampal-dependent spatial acquisition task, 3-month-old rats performed better than 18-month-old rats, in general. Our data corroborates previous studies describing age-related cognitive decline in Fischer rats (Gage et al., 1988; Markowska et al., 1989; Rapp and Amaral, 1992; Gallagher et al., 1993; Frick et al., 1995). However, rats exposed to isoflurane/ N₂O anesthesia at 18-months-old demonstrated poor spatial learning performance at 3 months post-anesthesia, but not at two weeks, compared to age-matched naïve controls. Previously, spatial impairment was reported in 18-month-old Fischer 344 rats following isoflurane and isoflurane/N₂O anesthesia up to 2 months post-anesthesia (Culley et al., 2004), and with N₂O alone at two weeks post-anesthesia (Culley et al., 2007). It may seem unusual that impairment occurred only at the later time point in our studies. However, a different dose of anesthesia was administered and a different behavioral test was used in the current study compared to previous studies (Culley et al., 2004, 2007). Additionally, 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 our study mimics the clinical incidence of POCD in elderly surgical patients (Moller et al., 1998; Biedler et al., 1999). The delayed presence of spatial learning impairments demonstrated here may result in disruption in learning and memory signaling pathways that occurs over time.

A current hypothesis regarding the mechanism of isoflurane-induced cognitive impairment, suggests that anesthetics trigger a systemic inflammatory response via inflammatory cytokines and caspase 3 activation (Xie et al., 2006, 2008, Zhang et al., 2008; Wu et al., 2010). In contrast to this hypothesis, no change was detected in our study in activated caspase 3 (fragment 17 kD) between groups in the hippocampus or cortex. However, an age-related increase in activated caspase-3 between 18- and 21-month-old rats regardless of anesthesia treatment was detected. The differences between our results and previous cell culture studies may involve the inherent differences between studies done in culture versus those performed *in vivo*. It is important to consider that anesthetic exposure to cultured cells may involve different cellular mechanism such that cell death may be the ultimate outcome (Zhang et al., 2009). In our study, no evidence of neuronal cell loss was detected at 3 months post-anesthesia in the brains of rats exposed to anesthesia at 18-months of age compared to naïve age-matched controls, demonstrated in supporting material (Fig. S2). Anesthetic exposure in an organism, such as rodent or human, may not subject the brain to the same type of cellular injury as those observed *in vitro*. There are inherent differences between studies performed in culture and those performed in the intact organism that must be considered. Additionally, some studies demonstrated caspase activation (Xie et al., 2008) and increases in pro-inflammatory cytokines (Wu et al., 2010) in naïve mice following 2 hours of 1.4% isoflurane exposure *in vivo*. The discrepancy between our results and these studies may involve the time of assay, such that caspase activation and pro-inflammatory cytokines were detected within 24 hours of anesthetic exposure in the previous studies (Xie et al., 2008; Wu et al., 2010), whereas our protein lysates were assayed immediately following anesthesia and at 2 weeks and 3 months post-anesthesia. It is possible that these inflammatory profiles were not significantly elevated immediately following anesthetic exposure and had already returned to baseline levels by later time points. The cellular damage initiated by anesthetic exposure is likely multi-factorial. Inflammatory processes involving caspase 3 activation and cell death at early time points may contribute to anesthesia-induced cognitive deficits in older animals. However, anesthetics may also initiate distinct cellular mechanisms that may have deleterious cellular effects, manifested at later times through a disruption of learning and memory signaling cascades.

We hypothesized that a disruption in NMDA-mediated signaling results in exposure of isoflurane/ N₂O anesthesia in the rat brain that was exposed to anesthetic at 18-months-old *in vivo*. Previously, an increase in NR2B subunit protein expression in the hippocampus of 4-5 month-old mice 24h after isoflurane anesthesia treatment was reported (Rammes et al., 2009). We report similar findings here in 18-month-old rats; with immediate and late (3 months post-anesthesia) increases in NR2B protein expression levels in the cortex and hippocampus of anesthesia-treated 18-month-old rats. One explanation for the subunit specific effects of isoflurane/N₂O anesthetic may involve the sensitivity of NMDA receptor subunits to isoflurane. Previously, in cortical neurons, NMDA-gated currents mediated by NR2B-containing receptors were more sensitive to isoflurane than currents mediated by NR2A-containing receptors (Ming et al., 2002). Additionally, subunit-specific changes in the brain occur with age, such that differences in behavioral outcomes have been demonstrated dependent on the levels of the NR2B subunit. Twenty-six-month-old mice with higher levels of NR2B demonstrate poor behavioral outcomes compared to age-matched controls (Zhao et al., 2009). In our study, the NMDA receptor was continuously antagonized by isoflurane for 4 hours of anesthesia treatment. It is a well studied

phenomenon that continuous antagonism of NMDA receptors causes a compensatory upregulation of these receptors (Gunduz-Bruce, 2009) with increased density of NMDA receptors measured by radioligand-binding studies (Aarons et al., 1980; Williams et al., 1992; Jenner et al., 1983). This upregulation makes neurons bearing the receptors more vulnerable to the excitotoxic effects of glutamate after the clearance of the anesthetic (Wang and Slikker, 2008). The increased sensitivity of NR2B-containing NMDA receptors to isoflurane antagonism (Ming et al., 2002) may explain the preferential increase in NR2B protein expression demonstrated here. Behaviorally, elevated levels of NR2B protein expression in the aged brain correspond to poor behavioral outcomes in aged mice (Zhao et al., 2009). Therefore, our findings suggests that the postponed upregulation of NR2B protein levels in the hippocampus and cortex observed at 3 months post-anesthesia correspond to spatial learning deficits observed in rats treated with anesthesia at 18-months-old. One explanation includes the possibility that the increase in NR2B protein expression may enhance long term depression (LTD). Previously, Liu and colleagues (2004) used ifenprodil and Ro 25-6981, NR2B subunit-selective NMDA receptor antagonists, and abolished induction of LTD in hippocampal slice preparations. Another explanation may involve a change in NR2B subunit protein expression shift to more extra-synaptic localization of NR2B subunit. Relocalization of NR2B-containing NMDA receptors is linked to impaired LTP (Gardoni et al., 2009), an increase in LTD (Massey et al., 2004) and/or increased 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 potential cell death (Hardingham et al., 2002). However, we were unable to detect anesthesia-induced cell loss in 18-month-old rats at 3 months post-anesthesia, shown in supporting material (Fig. S2). The involvement of NR2B and LTD in the aged brain in poor behavioral outcomes following anesthetic exposure deserves further investigation.

Also, the long-term 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 reduction in phospho-ERK1/2 levels 3 months post-anesthesia in the hippocampus and cortex of rats treated with anesthesia at 18-months-old. The reduction in phospho-ERK1/2 corresponded to spatial learning deficits in these animals. 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, corresponding to delayed spatial learning deficits in 18-month-old rats treated with anesthesia. Disruption of the NMDA receptor signaling through changes in NR2B may also interfere with other important learning and memory mechanisms, such as CaMKII activation (Santucci and Raghavachari, 2008; Pradeep et al., 2009) and AMPA receptor trafficking and regulation (Hall et al., 2007) that may offer additional explanations for the observed impairment in spatial learning in anesthesia-treated 18-month-old rats in this study.

In a separate experiment, we attenuated the anesthesia-induced acute NR2B specific elevation in the hippocampus of 18-month-old rats by pre-treating with the selective NR2B antagonist, Ro 25-6981 (Ro). 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) with IC₅₀ values of 0.009 of NR2B-containing receptors and 52μM for NR2A-containing receptors, *in vitro* (Fischer et al., 1997). The mode of action of Ro at NMDA receptors is similar to that of ifenprodil (Fischer et al., 1997), which is characterized as an “activity-

dependent” blocker (Kew et al., 1996) 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., 2009), Parkinson's disease (Loschmann et al., 2004), and neuropathic pain (Chizh et al., 2005). Continual antagonism of NMDA receptors results in upregulation of the receptor *in vitro* (Gunduz-Bruce, 2009). Therefore, the continual antagonism of NMDA receptors by isoflurane/N₂O anesthesia may explain the observed acute increase in NR2B protein expression. Pretreatment of anesthetized animals with Ro inhibited acute upregulation of NR2B in the hippocampus, suggesting that NMDA receptor antagonists may provide a promising therapeutic treatment against POCD. Further studies with Ro are warranted.

Careful interpretations of these data are necessary due to various limitations of this study, including: the spatial learning variability of 18-month-old rats, consideration of difficulties with interspecies extrapolation, and high experimental dose and duration of the applied anesthetic. To avoid the potential confounding variable of pre-existing cognitive deficits, 18-month-old Fischer rats that exhibited pre-existing spatial learning deficits were excluded. Detection of anesthesia induced spatial learning deficits on already impaired rats would be impossible 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, non-excluded 18-month-old rats and therefore cannot be extrapolated to aged Fischer rats in general. Extrapolation of rats to humans provides another caveat to interpretation of the results presented in the current study. The applied concentration of 1.8% isoflurane with 70% N₂O was above 1 MAC (minimum alveolar concentration) for rats. Anesthetic concentrations at this level are not typically used clinically. However, often high levels of anesthetics are used in experimental studies to determine underlying cellular mechanisms that may be subtle or concealed with clinically relevant doses. Various physiological parameters were measured during anesthetic exposure in 3- and 18-month-old rats, including: MAP, pH, pO₂, and pCO₂ shown in supporting material (Table S1). All parameters were maintained within normal physiological limits to confirm that animals were not hypoxic during the procedure and that rats maintained stable blood pressure during anesthetic exposure. The 4 hour duration of anesthesia for rats used in this study, extrapolated to humans by means of average lifespan, would be considerably longer than a normal surgical procedure. However, in a pilot study, we tested various lengths of this anesthetic dose (2h, 4h, and 6h; n=5/group). Four of the 5 18-month-old rats exposed to 6 hours of anesthesia expired during or shortly after anesthetic exposure. Four hours of anesthetic exposure was chosen because only the 4 hour duration resulted in significant behavioral deficits in 18-month-old rats that survived anesthetic treatment shown in supporting material (Fig. S3).

It is important to note that various evidence for mechanisms underlying neurocognitive impairment following anesthetic exposure has been previously described, attributing the deleterious effects of anesthesia to alteration of intracellular calcium homeostasis (Wei et al., 2008), enhanced neurodegenerative mechanism and neuroinflammatory cascades (Wan et al., 2007; Xie et al., 2008; Wu et al., 2010), and suppression of neural stem cell proliferation and migration in the aged brain (Stratmann et al., 2009; Sall et al., 2009). Our proposed mechanism involving the effect of isoflurane/N₂O anesthesia on NMDA receptors represents only one potential mechanism contributing to the impairment of spatial learning performance in 18-month-old rats. Age-related functional changes in the NR2B subunit of the aged brain produce vulnerabilities that may lead to unfavorable post-anesthesia consequences for older rats. Taken together, our results indicate a role for an elevation in NR2B protein expression following isoflurane/N₂O anesthesia in the 18-month-old rat brain, corresponding to spatial learning deficits in these animals. We also suggest a therapeutic intervention of this mechanism for inhibition of NR2B protein expression increases in the

hippocampus, with Ro 25-6981. However, further behavioral studies are required to demonstrate the efficacy of Ro pretreatment for spatial learning impairment in 18-month-old Fischer 344 rats. Although Ro is not approved for use in humans, our results suggest that treatments with NMDA receptor antagonists, such as Memantine for off-label treatment to prevent POCD, hold promise in future clinical studies.

4. Experimental Procedure

4.1 Animals

Experimental procedures were in compliance with *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, approved by the University of Miami Animal Care and Use Committee. Male Fischer rats (3-months-old; 250-280 g and 18 months; 420-480 g) were acquired from Charles Rivers laboratory the National Institute on Aging Colony at Harlan, respectively. Eighteen-months-old was chosen to represent “aged” rats because Fischer rats have a median life expectancy of 26 months, and have been shown to demonstrate age-related cognitive impairment (Frick et al., 1995) similar to the clinical population. Animals were kept on 12:12 h light: dark cycle, with access to food and water *ad libitum*.

4.2 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 (N=15 3-month-old; N=27 18-month-old rats). Animals were anesthetized with 3% isoflurane, 70% N₂O, and 30% O₂, 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 N₂O/O₂ mixture) lasted 4h. Blood gases, MAP, whole body and brain temperatures were monitored and maintained at physiological levels throughout anesthetic exposure. Temperatures were maintained at 37°C ± 0.5°C by heating lamps. After 4hrs, anesthesia was discontinued and, depending on the experimental condition, the animal was decapitated or ventilated on 30% O₂/70% 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.

4.3 Spatial learning behavioral testing

Animals were tested in spatial reference memory task before anesthetic exposure for baseline testing and during two testing sessions after anesthetic exposure. Naïve animals were tested at the same time as experimental counterparts. The spatial reference memory version of 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 a water maze navigational task. 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 NE quadrant for baseline 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, animals were tested over 3 days one week prior to anesthesia exposure. Each day, animals were given four trials. The animal was placed randomly at each of four starting points (north, south, east, and west)

and allowed 60 s to find the hidden platform. If the animal did not locate the platform within 60 s it was placed on it. The animal was allowed to remain on the platform for 30 s. After 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 (Gage et al., 1988; Markowska et al., 1989; Rapp and Amaral, 1992; Gallagher et al., 1993; Frick et al., 1995). Rats that received an average day 3 latency to platform score >30s were considered impaired and excluded from further testing. Non-excluded 18-month-old 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 18-month-old rats. Spatial acquisition learning was assessed at post-anesthesia days 15-17 and 90-92. The hidden platform was placed in the SE and SW quadrants for the two testing points, respectively. Latency to platform, path length, and swim speeds were measured for each rat. Individual scores were compared to baseline scores. The researcher who performed the behavioral tests and analysis was “blind” to the condition of the animal.

4.4 Antibodies and immunoblotting

Mouse monoclonal antibodies against NR2B (1:1000, Cell Signaling Technologies), and β -actin (1:5000, Sigma), polyclonal antibodies against NR2A, Caspase 3, pERK1/2, and ERK1/2 (all polyclonal antibodies at ratio 1:1000 from Cell Signaling Technologies) were used. Animals were terminated by decapitation immediately following anesthesia, 24h post-anesthesia, and after behavioral testing (2 weeks or 3 months post-anesthesia). Naïve age matched rats were terminated at the same times. Immunoblotting was performed as previously described (de Rivero Vaccari et al., 2008). Briefly, 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 $12,000 \times g$ for 3 min, and 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 difluoridemembranes (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 horseradish peroxidase (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. Data were normalized to β -actin.

4.5 Neuronal cell counts

For immunocytochemical studies, animals were re-anesthetized with 3% isoflurane N_2O/O_2 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 μ m thick were taken at 300- μ m intervals. 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 μ m 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-Imidoazole Buffer (pH 7.2), and then reacted with NiDAB (2.5% Nickel Ammonium Sulfate Acetate-Imidoazole 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 × 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. Animals were coded by number for analysis, and the investigator that performed cell counts was “blind” to the condition of the animal. 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 the all sections summed.

4.6 Ro 25-6981 Treatment

Ro 25-6981(Ro) molecular formula: C₂₂H₂₉NO₂-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, non-excluded 18-month-old rats (N=20) were randomly assigned to one of the following groups: Ro pretreatment followed by 4h anesthesia treatment, vehicle pretreatment with 4h of anesthesia treatment, Ro pretreatment with no anesthesia, or 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 terminated by decapitation (total time 4h 45min). Naïve rats were injected with Ro or saline, kept in the same room and terminated at the same time as anesthesia treated rats.

4.7 Statistical analysis

Healthy, non-excluded animals were randomized to experimental and control groups. Data are expressed as mean $\pm$ standard deviation ($\pm$ SD). Statistical comparisons between experimental and control groups were made using one-way analysis of variance (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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Spatial learning acquisition water maze performance in 18-month-old rats following isoflurane/ N₂O anesthesia treatment

(A) Latency to platform was measured at three testing points: baseline (days -7 to -5), postanesthesia 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) 18-month-old 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) 18-month-old 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 deviation (SD), n=12-15/group. Repeated measures of analysis of variance were performed, *p<0.05, **p<0.005, ***p<0.001)].

Fig. 2. Age-related increase in caspase 3 activation in the 18-month-old rat cortex and hippocampus

Representative immunoblots for (A) hippocampal and (B) cortical protein lysates from the brains of 18-month-old rats in the following experimental groups (a diagram of experimental groups is available in supporting material Fig. S1): Rats were non-treated or naïve at 18 months and terminated at 18 months (N (18m)) or 3 months later (N (+3m)), or anesthesia-treated at 18 months, then terminated immediately following anesthesia treatment (An (18m)) or terminated at 2 weeks post-anesthesia (An (+2wk)) or terminated 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 SD, $n=4-5$ /group. Oneway ANOVA and Tukey's post-hoc analysis were performed, $*p<0.05$, $**p<0.005$].

Fig. 3. Effect of isoflurane/ N₂O anesthesia on early and late protein expression of NMDA receptor subunits in the hippocampus and cortex of 18-month-old rats

(A) Representative immunoblots for hippocampal and (B) cortical protein lysates from the brains of 18-month-old rats in the following experimental groups (a diagram of experimental groups is available in supporting material Fig. S1): Non-treated or naïve at 18 months and terminated at 18 months (N (18m)) or 3 months later (N (+3m)), or anesthesia-treated at 18 months, then terminated immediately following anesthesia treatment (An (18m)) or terminated at 2 weeks post-anesthesia (An (+2wk)) or terminated 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 SD, n=4-5/group. One-way ANOVA and Tukey's post-hoc analysis were performed, *p<0.01, **p<0.005, ***p<0.001].

Fig. 4. Effect of isoflurane/ N₂O anesthesia on ERK1/2 activation in the hippocampus and cortex of 18-month-old rats

(A) Representative immunoblots for hippocampal and (B) cortical protein lysates rats exposed to isoflurane/N₂O anesthesia treatment or naïve at 18-months-old in the following experimental groups (a diagram of experimental groups is available in supporting material Fig. S1): Non-treated or naïve at 18 months and terminated at 18 months (N (18m)) or 3 months later (N (+3m)), or anesthesia-treated at 18 months, then terminated immediately following anesthesia treatment (An (18m)) or terminated at 2 weeks post-anesthesia (An (+2wk)) or terminated 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 SD, 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].

Fig. 5. Ro 25-6981 pre-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/N₂O 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 SD., $n=5-7$ /group. One-way ANOVA and Tukey's post-hoc analysis were performed, * $p<0.05$, n.s. =non-significant].