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Caffeine Combined with Sedative/Anesthetic Drugs Used in Neonatal Medicine and Apoptotic Neurotoxicity in Developing Mouse Brain

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Caffeine Combined with Sedative/Anesthetic Drugs Used in Neonatal Medicine and
Apoptotic Neurotoxicity in Developing Mouse Brain

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TABLE OF CONTENTS

| | |
|--|-------------------------------------|
| ABSTRACT | 3 |
| INTRODUCTION | ERROR! BOOKMARK NOT DEFINED. |
| Epidemiology of Preterm Birth | 4 |
| Complications Associated with Prematurity | 6 |
| Physiologic Apoptosis | 8 |
| Drug-Induced Developmental Apoptosis | 8 |
| Does SAD-induced Developmental Neurotoxicity Happen in Human Infants? | 13 |
| Caffeine as a Potential Apoptogen in Developing Brain | 14 |
| METHODS | 16 |
| Animals | 16 |
| Materials | 17 |
| Drugs. | 17 |
| Ketamine. | 17 |
| Fentanyl. | 18 |
| Caffeine. | 19 |
| Procedures | 19 |
| PND3 Experiments. | 19 |
| Multiple Exposure Experiments. | 20 |
| Single exposure (PND6) group. | 21 |
| Histopathology. | 21 |
| Quantitative Cell Counts. | 23 |
| Statistical Methods. | 23 |
| Hypotheses | 24 |
| RESULTS | 25 |
| Observations After Treatment | 25 |
| Experiment 1: Apoptogenic Action of CAF + Midazolam at PND3 | 25 |
| Experiment 2: Apoptogenic Action of CAF + Ketamine at PND3 | 26 |
| Experiment 3: Apoptogenic Action of CAF + Fentanyl at PND3 | 26 |

| | |
|--|-----------|
| Composite Dataset of the Apoptogenic Action of CAF + SADs at PND3 | 27 |
| Experiment 4: Apoptogenic Action of Single versus Multiple Exposures of CAF + Midazolam | 28 |
| Experiment 5: Apoptogenic Action of Single versus Multiple Exposures of CAF + Ketamine | 29 |
| Experiment 6: Apoptogenic Action of Single versus Multiple Exposures of CAF + Fentanyl | 29 |
| DISCUSSION | 30 |
| Interactions with Other NICU Drugs | 33 |
| Conclusion | 35 |
| REFERENCES | 37 |
| FIGURES | 55 |

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Caffeine Combined with Sedative/Anesthetic Drugs Used in Neonatal Medicine and
Apoptotic Neurotoxicity in Developing Mouse Brain

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Abstract

Each year, millions of premature babies are exposed to sedative/anesthetic drugs (SADs) in the neonatal intensive care unit (NICU). Acute exposure to SADs triggers widespread apoptosis in the developing brain of rodents and non-human primates. Furthermore, premature infants are administered caffeine (CAF) to treat respiratory dysfunction. Mounting evidence suggests that CAF may be neurotoxic and, when given in combination with SADs, potentiates SAD-induced cell death. However, the apoptotic interaction of CAF and SAD co-exposure is poorly understood. In a series of studies, I report that CAF combined with the NICU SADs midazolam, ketamine, or fentanyl is more neurotoxic to the postnatal day 3 (PND3) mouse brain than either CAF or SAD alone. Since many premature infants are given CAF + SADs chronically, I tested whether multiple exposures of CAF + SAD at PND3 + PND6 is more detrimental to the developing brain than a single exposure at PND6. My results indicate that the PND6 mouse brain is as vulnerable to multiple exposures of CAF + SAD on PND3 and PND6 as it is to a single exposure on PND6. Based on these results, neonatologists should exercise caution by limiting CAF + SADs co-exposure to durations necessary to ensure the survival of babies born prematurely.

Introduction

Epidemiology of Preterm Birth

In 2010, 15 million infants were born prematurely across the world (Blencowe et al., 2012). Complications from being born too early are the leading cause of death amongst neonates, accounting for approximately 35% of 3.1 million deaths and the second leading cause of mortality after pneumonia in children under 5 years of age (Liu et al., 2015). Although one million premature infants are estimated to die annually, the vast majority of babies survive into the post-neonatal period. However, achieving such an impressive survival rate often requires aggressive medical intervention and days, weeks, or even months in the neonatal intensive care unit (NICU). As preterm birth rates continue to rise in nearly every country with reliable data – especially high-income countries like the United States – more infants will need such care (Blencowe et al., 2012). Being born prematurely taxes medical resources and incurs significant financial costs to the parents of preterm infants. Families also must deal with the emotional burden of potentially lengthy NICU stays, and many preterm infants have perinatal medical conditions that require immediate treatment at birth. A significant subset will have neuromotor and behavioral morbidities, setting the stage for poor quality of life and a lifetime of disability (Blencowe et al., 2012). Thus, the public health burden of prematurity is expansive, with significant medical, economic, emotional, and societal consequences.

The World Health Organization (WHO) defines preterm birth as being born before 37 weeks of gestation (Lawn et al., 2013). Prematurity is subdivided further into

categories based on gestational age: moderate-to-late preterm (32 to 36 weeks); very preterm (28 to 31 weeks); and extremely preterm (less than 28 weeks). Complications due to prematurity increase with decreasing gestational age since very preterm and extremely preterm infants are born with underdeveloped physiological systems that are not prepared for life outside the womb. Importantly, stratifying preterm birth by gestational age – as well as birth weight – allows neonatologists to anticipate potential complications and deliver immediate and appropriate care.

According to WHO data, only 10 countries are responsible for over 60% of preterm births globally (Blencowe et al., 2012). Notably, the United States (ranked 6th) is the only high-income country on the list. This somewhat dubious distinction is important because middle- and low-income countries typically lack basic medical infrastructure to care for preterm infants, and many of these babies die before reaching the post-neonatal period. In contrast, the United States has sophisticated medical infrastructure capable of saving the lives of many preterm infants, even those of extremely low gestational age with major medical morbidities. This situation is a double-edged sword given that mortality due to premature birth is low in the United States, yet the large numbers of babies that do survive are at increased risk for neuropathological outcomes and life-long neurodevelopmental impairment (NDI). However, not all premature infants have NDI, which begs the question why some are at risk for NDI while others are not.

Complications Associated with Prematurity

Recent advances in intensive care have reduced premature infant mortality significantly in the last decade (Horbar et al., 2012). However, complications associated with prematurity represent a unique set of challenges for neonatologists. Premature infants typically present to the NICU with comorbid conditions that require immediate and aggressive medical intervention. Babies born too soon are at risk for hypoxic-ischemia, intraventricular hemorrhaging, and necrotizing enterocolitis, complications that can be debilitating or rapidly fatal (Ward & Beachy, 2003). Thus, during their stay in the NICU, preterm infants frequently undergo invasive and painful medical procedures including intravenous or arterial catheterization, blood sampling, and major surgeries (Barker & Rutter, 1995; Carbajal et al., 2008).

Furthermore, respiratory dysfunction is ubiquitous in preterm infants and may be present even in full-term neonates (Ward & Beachy, 2003). Immaturity of central and/or peripheral systems at birth contributes to poor respiratory drive. Premature infants often suffer from respiratory distress syndrome, a consequence of insufficient lung surfactant production and a leading cause of mortality in premature babies (Barton, Hodgman, & Pavlova, 1999). Central apnea of prematurity, defined as cessation of breathing for 20 seconds or more, is caused by immature brain substrates controlling respiration, particularly the medulla oblongata (Gerhardt & Bancalari, 1984; Martin & Abu-Shaweesh, 2005; Martin & Wilson, 2012). Apnea of prematurity is accompanied by episodes of hypoxia and bradycardia that occur sporadically or chronically during extended hospital stays (Henderson-Smart & Steer, 2001; Schmidt et al., 2006; Zhao, Gonzalez, & Mu, 2011). Many infants also are mechanically ventilated to treat

respiratory dysfunction and, therefore, at risk for bronchopulmonary dysplasia caused by long-term intubation (Kinsella, Greenough, & Abman, 2006). With each of these disorders, regimens of glucocorticoids and/or central nervous system stimulants are indicated to accelerate pulmonary maturation and enhance respiratory drive.

Within the last two decades, clinicians and researchers have recognized that medical morbidities are not the only sequelae premature infants experience. Indeed, being born too soon is a risk factor for NDI that persists into childhood and beyond (Bhutta, Cleves, Casey, Craddock, & Anand, 2002; Marret et al., 2013). Compared to full term cohorts, premature infants tend to have lower IQ scores, as well as memory and attention deficits (Skranes et al., 2013). Furthermore, prematurity is associated with poor behavioral and emotional control, language delay, and deficient visual and auditory processing (Sølsnes, Skranes, Brubakk, & Løhaugen, 2014). Not surprisingly, neurodevelopmental sequelae associated with prematurity are inversely proportional to gestational age, with extremely preterm infants at greatest risk for NDI (Lawn et al., 2013).

Neuroimaging studies provide insight into the underlying neuropathology that may contribute to preterm NDI. White matter injuries in brain regions involved in cognition – cortex, basal ganglia, and cerebellum – are common in the premature infant population (Volpe, 2009). More recently, reports have emerged that prematurity is associated with decreased cortical and deep nuclear gray matter volume, loss of white matter integrity, ventricular dilation, and poor overall brain connectivity (Bjaland, Rimol, Løhaugen, & Skranes, 2014; Eikenes, Løhaugen, Brubakk, Skranes, & Haberg, 2011; Eikenes et al., 2012; Filan, Hunt, Anderson, Doyle, & Inder, 2011; Skranes et al., 2013).

Yet, not all preterm infants are at risk for neuropathology and NDI, even when compared to cohorts of similar gestational age (Larroque et al., 2008). Precisely why a subset of these babies is prone to stunted neurodevelopment despite aggressive and successful medical intervention is poorly understood. A working hypothesis derived from a growing body of research is that exposure to several classes of drugs used in neonatal medicine may be partially responsible for neuropathological outcomes and NDI in infants born too soon.

Physiologic Apoptosis

Mammalian brain development follows an orchestrated program reliant on cells establishing proximal and distal synaptic connections and receiving neurotrophic signals that promote their survival. This period of synaptogenesis extends from mid-gestation through the first few years of life in humans and primarily occurs within the first two weeks of life in rodents (www.translatingtime.net). Cells that do not receive neurotropic support or fail to make connections with other cells commit suicide via apoptosis (programmed cell death). This background level of cell death (physiologic apoptosis) prunes redundant or dysfunctional cells and is necessary to sculpt the brain into a highly-connected, resource-efficient organ (Dikranian et al., 2001). Thus, synaptogenesis and physiologic apoptosis work in concert to establish the cytoarchitecture of the brain.

Drug-Induced Developmental Apoptosis

Synaptogenesis also represents a window of vulnerability during which exposure of the developing brain to several classes of drugs causes apoptotic neurotoxicity: the deletion of millions of cells that would otherwise contribute to normal brain connectivity

and function. Drugs that induce developmental neurotoxicity include glutamate *N*-methyl-*D*-aspartate (NMDA) receptor antagonists, γ -amino-butyric acid receptor subtype A (GABA_A) agonists, and ethanol, which possess both NMDA receptor antagonist and GABA_A agonist properties (Olney et al., 2002). Many neurotoxic drugs may be abused by pregnant mothers – ethanol, phencyclidine, benzodiazepines – or are anti-epileptic drugs used to manage seizures in infants and children, e.g., phenobarbital, valproate (Bittigau et al., 2002; Farber & Olney, 2003; Ikonomidou et al., 1999; Ikonomidou et al., 2000).

Particularly troubling is that sedative/anesthetic drugs (SADs) common in obstetric and pediatric medicine may be neuropathogens. Given that preterm neonates undergo multiple invasive procedures while hospitalized (Barker & Rutter, 1995), SADs have been invaluable in the NICU to immobilize the patient, manage pain, and control sympathetic nervous system responses. Many SADs are considered safe and beneficial, a reputation based on the absence of deleterious side effects rather than *a priori* testing in, and approval for, neonatal and pediatric populations. However, the United States Food and Drug Administration has not approved the majority of drugs employed in the NICU for use in pediatric settings (Barr et al., 2002; Clark, Bloom, Spitzer, & Gerstmann, 2006). Importantly, most, if not all, NICU SADs have either NMDA antagonist or GABA_A agonist properties and are potentially neurotoxic to human infants.

Indeed, acute exposure of the developing brain to SADs during synaptogenesis triggers widespread apoptotic neurotoxicity in rodents and non-human primates. Ikonomidou and colleagues (1999) first documented that ketamine, an NMDA receptor antagonist, induced robust apoptotic neurotoxicity in postnatal day (PND) 7 rat pups

throughout the extent of the neuraxis. This initial observation was extended in a series of studies testing several classes of GABA-mimetic SADs frequently used in perinatal medicine such as inhalation anesthetics and benzodiazepines. Jevtovic-Todorovic et al. (2003) reported that brief (6 hour) exposure to the inhalation anesthetic isoflurane caused dose-dependent apoptotic neurotoxicity in developing rat brain, while Young et al. (2005) found that a single, sub-sedative/anesthetic dose of midazolam was neurotoxic to PND7 mice.

In the wake of these seminal studies, researchers have built an extensive body of evidence suggesting that nearly all SADs tested are neurotoxic to the developing mammalian brain. Notably, these agents are neurotoxic *at or below* doses and exposure durations that are used clinically in human infants. For example, brief exposure to isoflurane or its structural analogues, sevoflurane and desflurane, was equally neurotoxic to the PND7 mouse brain (Istaphanous et al., 2011), while Johnson, Young, and Olney (2008) found that even transient (2 hour) exposure to a sub-anesthetic concentration of isoflurane is sufficient to cause neurotoxicity in neonatal rodents. Independent research groups have documented that sub-sedative/anesthetic doses of ketamine, midazolam, diazepam, and propofol consistently trigger a widespread apoptotic response in developing rodent brain (Bittigau et al., 2002; Cattano, Young, Straiko, & Olney, 2008; Fredriksson, Archer, Alm, Gordh, & Eriksson, 2004; Fredriksson, Ponten, Gordh, & Eriksson, 2007; Scallet et al., 2004; Stevens et al., 2011; Yuede, Olney, & Creeley, 2013; Young et al., 2005; Zou, Patterson, Sadovova, et al., 2009).

The developing non-human primate brain also is vulnerable to apoptotic neurotoxicity after SAD exposure. Brambrink and colleagues (2010) exposed PND6

rhesus monkeys to an anesthetic plane of isoflurane for 5 hours and observed a 13-fold increase in apoptotic neurotoxicity compared to control animals. The distribution of apoptotic cells was spread throughout neocortex and subcortical structures, as well as white matter tracts. In subsequent studies, the authors confirmed that oligodendrocytes are indeed susceptible to isoflurane- and propofol-induced apoptosis (Brambrink et al., 2012; Creeley et al., 2013). Furthermore, maternal exposure to an anesthetic plane of propofol for 5 hours caused a massive apoptotic response in both neurons and oligodendrocytes of fetal rhesus monkeys (Creeley et al., 2013). Thus, maternal exposure to SADs is likely detrimental to the developing brain *in utero*, a hypothesis supported by independent research groups that administered anesthetic concentrations of ketamine to pregnant rhesus monkeys (Slikker et al., 2007; Zou, Patterson, Divine, et al., 2009).

Based on these studies, it appears that two major cell types – neurons and oligodendrocytes – are susceptible to SAD-induced apoptosis. Cerebrocortical neurons are particularly vulnerable, as are neurons in thalamus, caudate putamen, superior and inferior colliculus, and hippocampus. Furthermore, both premyelinating and myelinating oligodendrocytes disintegrate after SAD exposure, particularly in corpus callosum, coronal radiata, internal capsule, and cerebellar peduncles (Brambrink et al., 2012; Creeley et al., 2013). Given the phylogenetic similarity of non-human primates to humans, these findings are particularly troubling since: 1. Maternal exposure to SADs may impact fetal brain development via apoptotic neurotoxicity and 2. Exposure to SADs may be a dual insult that deletes millions of neurons and oligodendrocytes while impairing the ability of surviving neurons to communicate effectively.

With the loss of millions of cells that would otherwise contribute to normal brain connectivity and function, an expected outcome of SAD-induced apoptotic neurotoxicity is behavioral disturbances in later life. Indeed, developing animals exposed to SADs exhibit long-term NDI that manifests as deficits in numerous domains associated with normal brain function. A clinically-relevant anesthetic cocktail of isoflurane, midazolam, and nitrous oxide administered to PND7 rats caused long-term (reference) memory deficits and impaired working memory capacity that persisted well into adulthood (Jevtovic-Todorovic et al., 2003). When tested as juveniles, rats exposed to a low concentration of isoflurane on PND7 displayed decreased freezing time in a contextual fear paradigm, suggesting abnormal hippocampal function (Sanders et al., 2009). Additionally, mouse pups exposed to ketamine, ketamine and diazepam, or ketamine and propofol exhibited hyperactivity and poor learning as adults, indicative of attentional disturbances and persistent memory impairment (Fredriksson et al., 2004; Fredriksson et al., 2007; Viberg, Ponten, Eriksson, Gordh, & Fredriksson, 2008). Paule and colleagues (2011) administered anesthetic levels of ketamine to PND5 and PND6 neonatal rhesus monkeys and charted their neurodevelopment longitudinally. Around 10 months of age, ketamine exposed animals had poorer performance on measures of short-term memory, psychomotor speed, and learning compared to control animals. Importantly, poorer performance on these tasks persisted at 3 years of age in ketamine-exposed animals. Taken together, these studies suggest that acute exposure to SADs during mammalian brain development may result in long-lasting NDI.

Does SAD-induced Developmental Neurotoxicity Happen in Human Infants?

The findings summarized above are relevant in a public health context since millions of infants and children worldwide are exposed to SADs each year. A growing body of clinical evidence supports the hypothesis that SADs are neurotoxic to the developing human brain and may contribute to NDI. Fetal exposure to ethanol, which has GABA_A agonist and NMDA antagonist properties, results in well-characterized neuropathological changes and long-term NDI that manifest as fetal alcohol spectrum disorder (Farber, Creely, & Olney, 2010; Ikonomidou et al., 2000; Mattson, Crocker, & Nguyen, 2011; Olney, 2004; Wozniak et al., 2004). Furthermore, *in utero* exposure to GABAergic anti-epileptic drugs such as valproate and carbamazepine is associated with long-term NDI (Banach, Boskovic, Einarson, & Koren, 2010; Meador et al., 2009; Meador et al., 2012). Finally, several clinical studies have reported that exposure of human neonates to SADs significantly increases the risk of neuropathology and NDI compared to non-exposed cohorts (DiMaggio, Sun, Kakavouli, Byrne, & Li, 2009; DiMaggio, Sun, & Li, 2011; DiMaggio, Sun, Ing, & Li, 2012; Flick et al., 2011; Ing et al., 2012; Sprung et al., 2012; Wilder et al., 2009). Particularly troubling is that abnormal outcomes in behavioral, linguistic, attentional, and higher-order cognitive domains in SAD-exposed infants are markedly similar to those seen in preterm infants (Flick et al., 2011).

The best evidence to date bridging the gap between preterm infant NDI and SAD-induced neurotoxicity comes from studies conducted by Filan and colleagues (2012) and Morriss et al. (2014). In both studies, very preterm infants were classified broadly as non-SAD-exposed or SAD-exposed. In a large, multicenter study funded by the National

Institute of Child Health and Human Development, Morriss et al. (2014) found that 50% of preterm infants undergoing major surgeries requiring general anesthesia experienced cognitive and psychomotor NDI at 18 to 22 months of age. In striking similarity to neuropathology associated with prematurity, Filan et al. (2012) reported that exposure to SADs was a significant risk factor for white matter injury and decreased cortical and subcortical brain volume. Furthermore, at two years of age, premature infants administered SADs had poorer cognitive and psychomotor function compared to the non-exposed cohort. Notably, neuropathology and NDI associated with preterm infant SAD exposure map well onto the results from animal studies: decreased cortical/subcortical volume and white matter injury likely due to apoptotic death of millions of neurons and oligodendrocytes, with concomitant disturbances in cognition and psychomotor speed.

Caffeine as a Potential Apoptogen in Developing Brain

Given that respiratory dysfunction is ubiquitous in preterm infants, neonatologists correctly anticipate that babies born less than 32 weeks of gestational age will need respiratory stimulation to survive. In this context, caffeine (CAF) is considered a safe, beneficial, and cost-effective drug that stimulates respiratory drive with few adverse side effects (Aranda, Beharry, Valencia, Natarajan, & Davis, 2010; Doyle et al., 2010; Schmidt et al., 2006, 2007). While structural analogues such as aminophylline, theophylline, and theobromine have fallen out of favor due to safety concerns, CAF use in the NICU has surged, with Clark et al. (2006) reporting a 69% increase in CAF administration between 1999 and 2004. Indeed, CAF is currently the drug of choice to treat apnea of prematurity and improve respiratory drive during mechanical ventilation (Aranda, Beharry, Valencia, Natarajan, & Davis, 2010; Bauer et al., 2001). In the

absence of visibly toxic side effects, some researchers have wondered whether even monitoring CAF blood levels in premature infants is necessary (Aranda et al., 2010; Leon, Michienzi, Ma, & Hutchison, 2007; Natarajan, Botica, Thomas, & Aranda, 2007).

However, emerging evidence suggests that CAF may be a neuroapoptogen in developing mammalian brain. Kang and colleagues (2002) reported that high doses of CAF are neurotoxic to PND7 rat pups *in vivo* and to cultured fetal mouse neurons *in vitro*. More troubling is that, in contrast to its safe reputation, CAF may potentiate SAD-induced neurotoxicity in developing mouse brain. Black and colleagues (2008) reported that CAF co-administered with morphine caused more cell death in the PND3 rat brain than morphine alone. In a comprehensive set of studies, Yuede et al. (2013) tested CAF in combination with ethanol, GABA mimetics (diazepam, isoflurane), and NMDA antagonists (PCP, ketamine). In each case, CAF potentiated the apoptotic response to these agents. The pro-apoptotic action of CAF in combination with SADs is being replicated in the neonatal non-human primate brain with a similar outcome: an isoflurane and CAF cocktail was far more apoptogenic than isoflurane alone (Noguchi, K. K., personal communication).

Premature infants are often exposed to multiple drugs in the NICU for days, weeks, or months, and the neurotoxic consequences of these cocktails in the developing brain have not been studied in detail. Many babies born too soon are co-administered high doses of SADs and CAF to complete medical procedures and improve respiratory drive. In contrast to the dogma of safety in the NICU, translational research suggests that exposure to clinically-relevant doses of SADs is sufficient to trigger widespread apoptosis in rodents and non-human primates, with concomitant long-term

neuropathology and behavioral disturbances. Furthermore, CAF may be an apoptogen by itself and may potentiate SAD-induced neurotoxicity. Since millions of preterm infants are treated with SADs and CAF each year, exposure to these agents may partially explain why a subset of premature babies is at greater risk for neuropathology and NDI than others.

To better understand putative neurotoxic interactions with SADs, I tested CAF in combination with three of the most commonly used SADs in neonatal medicine: midazolam, ketamine, and fentanyl. My studies were designed to answer two primary research questions: 1. Does CAF have pro-apoptotic properties in the extremely immature (PND3) mouse brain when combined with midazolam, ketamine, or fentanyl? 2. Since many preterm infants are administered CAF + SADs chronically, are multiple exposures of CAF plus midazolam, ketamine, or fentanyl at PND3 + PND6 more neurotoxic than a single exposure at PND6?

Methods

Animals

In all experiments, I used litters of ICR mouse pups, and littermates were randomly assigned to experimental conditions. Dams and their litters were housed at 21 °C on a 12 hour light/dark cycle with *ad libitum* access to food and water. Pregnant mice were observed daily to ensure precise identification of birth date of litters. Day of birth was defined as PND0. All animal procedures were approved by the Washington University in St. Louis Animal Studies Committee.

Materials

Drugs. I chose to administer CAF in combination with three SADs that are popular in neonatal medicine based on their safety and efficacy in the NICU (Clark et al., 2006): midazolam, ketamine, and fentanyl. Pups were injected via the intraperitoneal (IP) route using a Hamilton syringe with a 27-gauge needle. *Midazolam.* Midazolam is a GABA_A agonist of the benzodiazepine class. Midazolam is a popular SAD in the NICU due to its short half-life and is often given as a sedative in conjunction with general anesthetics. Doses of midazolam for neonatal sedation range from 0.02 to 0.3 mg/kg/hour (Anand et al., 2009; Anderson & Larsson, 2011), while its ED₅₀ for loss of righting reflex in mice is approximately 40 mg/kg (Ben-Shlomo, Rosenbaum, Hadash, & Katz, 2001; Inada, Asai, Yamada, & Shingu, 2004). Thus, the sedating dose of midazolam in mice is approximately 133 to 2,000 times higher than in humans (40 mg/kg divided by 0.3 mg/kg/hour and 0.02 mg/kg/hour, respectively). Preservative-free midazolam hydrochloride solution (Abbott Laboratories, Chicago, IL, USA) was administered at 6 mg/kg for all experiments, which translates into a 0.02 to 0.003 mg/kg dose in humans (6 mg/kg divided by 133 and 2,000, respectively). Since midazolam is administered as an intravenous infusion to neonates, a 6 mg/kg dose in mice is well below the total doses that human infants may receive.

Ketamine. Ketamine is a non-competitive NMDA receptor antagonist and potent dissociative that induces sedation/anesthesia while maintaining respiratory reflexes. Ketamine hydrochloride was dissolved in sterile saline (Letco Medical, Decatur, AL, USA) and administered at 40 mg/kg in all experiments. In neonatal medicine, ketamine is typically administered as a bolus of 0.5 to 2 mg/kg every 10 minutes or at 0.3 to 1.2

mg/kg/hour as continuous infusion (Hall, 2012; Lexicomp Online, 2016; Lyon, Dabbs, & O'Meara, 2007; Pees, Haas, Ewert, Berger, & Lange, 2003). However, the ED₅₀ of ketamine to induce anesthesia in mice is approximately 80 mg/kg (Green, Knight, Precious, & Simpkin, 1981). Thus, an anesthetic dose of ketamine in mice is approximately 67 to 267 times higher in humans than in mice (80 mg/kg divided by 1.2 mg/kg/hour and 0.3 mg/kg/hour, respectively). The 40 mg/kg dose of ketamine used in the current experiments is the equivalent of a 0.6 mg/kg to 0.15 mg/kg dose for a human neonate (40 mg/kg divided by 67 and 267, respectively). Similar to midazolam, a 40 mg/kg dose in mice is substantially lower than the total dose a neonate would receive via continuous infusion of ketamine to induce anesthesia.

Fentanyl. Fentanyl is widely used in the NICU as a first-line sedative and general anesthetic. Fentanyl has unique pharmacodynamics compared to midazolam or ketamine in that its sedative/anesthetic effects are mediated by binding and activating G-protein coupled μ opioid receptors. For induction of anesthesia in neonates, fentanyl is administered as a bolus of 10 μ g/kg, while its ED₅₀ for anesthesia in mice is 60 μ g/kg (Gargiulo et al., 2012; Lexicomp Online, 2016; Yaster, 1987). Fentanyl sedation is accomplished by continuous infusion at a rate of 1 to 4 μ g/kg/hour (Anand, 2001; World Health Organization Guidelines for Pediatric Pain, 2012; Lexicomp Online, 2016). Thus, the anesthetic dose of fentanyl in mice is approximately 6 times higher than in human infants (60 μ g/kg divided by 10 μ g/kg). In all experiments, I used a 40 μ g/kg dose of fentanyl citrate (Hospira Inc., Lake Forest, IL, USA). Therefore, the chosen dose of fentanyl translates to a 6.7 μ g/kg in human infants (40 μ g/kg divided by 6), well below the threshold dose for anesthesia induction in neonates.

Caffeine. CAF is a centrally-acting stimulant that antagonizes G-protein coupled adenosine receptors. While the adenosine receptor subtype 1 (A₁) is inhibitory, the adenosine receptor subtype 2_A (A_{2A}) is excitatory and expressed abundantly on GABAergic cells in the medulla (Martin & Abu-Shaweesh, 2005). Thus, CAF acts as a respiratory stimulant by antagonizing A_{2A} receptors and increasing glutamate release in medulla via disinhibition. In all experiments, I used an 80 mg/kg dose of CAF (Sigma-Aldrich, St. Louis, USA) dissolved in saline. In the NICU, CAF is typically administered at a 20 mg/kg loading dose with maintenance doses in the range of 5 to 10 mg/kg/day (Schmidt et al., 2006). Blood levels of CAF in the range of 6 to 50 µg/mL are considered safe and therapeutic for premature infants (Natarajan et al., 2007). When administered to PND4 mice, an 80 mg/kg dose of CAF produces a mean blood level of 38 µg/ml (Yuede et al., 2013), which is well within the range considered safe for premature infants.

Procedures

PND3 Experiments. Midazolam. In experiment 1, litters of PND3 mice ($n \geq 4$ per group) received IP injection of vehicle or 6 mg/kg midazolam with or without an 80 mg/kg dose of CAF. Thus, experiment 1 had four groups: control (CON), midazolam (MIDAZ), CAF, or CAF + MIDAZ. **Ketamine.** In experiment 2, litters of PND3 mice ($n \geq 4$ per group) were injected IP with vehicle or 40 mg/kg ketamine with or without an 80 mg/kg dose of CAF. The four groups in experiment 2 were CON, ketamine (KET), CAF, or CAF + KET. **Fentanyl.** In experiment 3, litters of PND3 mice ($n \geq 4$ per group) received IP injection of vehicle or 40 µg/kg fentanyl with or without an 80 mg/kg dose of CAF. CON, fentanyl (FENT), CAF, or CAF + FENT comprised the four groups in experiment 3.

Since midazolam, ketamine, and fentanyl have short half-lives (Hijazi, Bodonian, Bolon, Salord, & Boulieu, 2003; Katz & Kelly, 1993; Wildt et al., 2001), pups assigned to receive these agents, either alone or in combination with CAF, were given a booster injection 3 hours after initial injection to maintain steady state levels of drug. Animals assigned to the CON or CAF groups were administered vehicle 3 hours after initial injections. During all experiments, pups were maintained in a V1200 Mediheat Veterinarian Recovery Chamber (Harvard Apparatus, Holliston, MA, USA) at 30 °C separate from the mother. Six hours following initial injections, animals were euthanized for histological analysis.

Multiple Exposure Experiments. *Multiple exposure (PND3 + PND6) group.*

Similar to the PND3 studies, I chose 6 mg/kg midazolam, 40 mg/kg ketamine, or 40 µg/kg fentanyl for these experiments. To test for differences in neurotoxicity between single or multiple exposure regimens, litters of PND3 mice ($n \geq 4$ per group) received IP injection of vehicle or SAD with or without an 80 mg/kg dose of CAF. Thus, the four treatments were CON, SAD, CAF, or CAF + SAD. Three hours after initial injection, pups assigned to the SAD or CAF + SAD groups were administered a booster dose of SAD, while those assigned to the CON or CAF groups received vehicle. During PND3 injections, pups were maintained in a V1200 Mediheat Veterinarian Recovery Chamber (Harvard Apparatus, Holliston, MA, USA) at 30 °C separate from the mother and returned to their home cage after booster injections. Pups were left undisturbed until PND6, when they received their respective treatment and booster again, were maintained in a recovery chamber separate from the mother, and euthanized 6 hours after initial

injections for terminal histology. These animals comprised the multiple exposure group (PND3 + PND6).

Single exposure (PND6) group. For the single exposure group, an independent set of litters was injected only on PND6 (PND6 group) with vehicle or SAD with or without 80 mg/kg CAF, creating four treatments: CON, SAD, CAF, and CAF + SAD. Animals in the PND6 group assigned to the SAD or CAF + SAD conditions received a booster dose of SAD 3 hours after initial injections, while those assigned to the CON or CAF conditions received vehicle. Pups were maintained in a recovery chamber at 30 °C separate from their mother until euthanasia 6 hours after initial injections.

Combining these two groups for statistical analyses yielded a 2 (Exposure: Single PND6 versus Multiple PND3 + PND6) x 4 (Treatment: CON, SAD, CAF, or CAF + SAD) factorial design for each SAD tested. Thus, I tested the pro-apoptotic action of single versus multiple exposures of 80 mg/kg CAF with 6 mg/kg midazolam (experiment 4), 40 mg/kg ketamine (experiment 5), and 40 µg/kg fentanyl (experiment 6).

Histopathology. Activated caspase 3 (AC3) is a sensitive histological marker of apoptosis. Commitment to apoptotic cell death occurs prior to activation of caspase 3, and immunolabeling for AC3-positive profiles is a reliable way to map and quantify dying cells that have already committed to suicide. For the PND3 experiments, all animals were euthanized 6 hours after initial injection on PND3. For multiple exposure experiments, all animals were euthanized 6 hours after initial injection on PND6. I chose 6 hours as the experimental endpoint because activation of caspase 3 is a transient phenomenon that takes approximately 2 hours to run its course in a given cell.

Furthermore, after drug challenge, maximum caspase 3 activation occurs earlier in some brain regions (~2 hours in cortex and caudate putamen) than others (~8 hours in thalamus; Johnson et al., 2008; Olney et al., 2002). Euthanasia at 6 hours after initial injections provides a snapshot of the average AC3 response throughout the extent of the neuraxis.

At the chosen experimental endpoint, animals were deeply anesthetized with a 70 mg/kg dose of sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA) fixative via the left cardiac ventricle and ascending aorta. Brains were postfixed in PFA for 24 hours at 4 °C, extracted the next day, and preserved in fixative at 4 °C until sectioning. Harvested brains were embedded in 3% agar and serially sectioned in the sagittal plane at 75 µm on a vibratome.

Every 8th section was subjected to immunohistochemistry for AC3 by methods previously described (Cabrera et al., 2014; Noguchi, Lau, Smith, Swiney, & Farber, 2011). Briefly, sections were washed in 0.01M phosphate-buffered saline (PBS) and 0.1% Triton X-100, quenched for 10 minutes in 3% hydrogen peroxide in methanol, and incubated for 1 hour in blocking solution (2% bovine serum albumin/0.2% milk/0.1% Triton X-100 in PBS). Sections were incubated overnight in rabbit anti-AC3 (Cell Signaling Technology, Danvers, MA, USA) at 1:1000 dilution in blocking solution. The following day, sections were incubated with goat anti-rabbit secondary (1:200 dilution) in blocking solution and reacted in dark with ABC reagents (Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 hour. AC3 positivity was visualized using VIP substrates for peroxidase (Vector Labs, Burlingame, CA, USA).

Quantitative Cell Counts. The regions maximally affected by NMDA antagonists, GABA_A agonists, and ethanol in the developing mouse brain include the subiculum and hippocampal formation, caudate putamen, retrosplenial cortex, thalamus, superior and inferior colliculi, and all major divisions of the neocortex. These brain areas comprised the regions of interest (ROIs) for all experiments, and apoptotic cell counts are presented as mean density per mm³ across all these regions.

Serial sectioning yielded 3 to 4 sagittal sections per animal encompassing the ROIs, and these sections were subjected to unbiased stereology via the optical fractionator method as previously described (Yuede et al., 2013). Briefly, the ROIs were outlined using Stereo Investigator software (MicroBrightField, Inc., Colchester, VT, USA) on a Pentium III PC connected to a Prior Optiscan motorized stage (ES103 XYZ system, Prior Scientific Inc., Rockland, MA, USA) with a Nikon Labophot-2 microscope. The population estimator function of Stereo Investigator was used to mark only AC3-positive profiles with visible processes or pyknotic soma while they were counted to ensure that no profile would be missed or counted twice. The estimated population of AC3-positive cells from each brain was divided by tissue volume to obtain density of AC3-positive cells per mm³. Density of AC3-positive profiles per mm³ was the primary dependent variable in analyses, and counts were performed by an observer blind to treatment.

Statistical Methods. Statistical analysis of PND3 AC3-positive density counts was performed via one-way ANCOVA with litter as a covariate to control for differences in physiologic (natural) apoptosis variability between litters. One-way ANCOVA was followed by Fisher's LSD post hoc analysis to determine differences in mean density of

apoptotic profiles per mm^3 between the CON, SAD, CAF, and CAF+ SAD groups. To test multiple exposures, P6 and P3 + P6 data were analyzed via a 2 (Exposure: Single vs. Multiple) x 4 (Treatment: CON, SAD, CAF, and CAF + SAD) factorial ANCOVA with litter as a covariate. Any significant Treatment x Exposure interactions were followed by simple main effects analysis to determine the group(s) driving the interaction. Positive results in all analyses were probed further with Cohen's d to determine effect size. Briefly, Cohen's d is the difference between two means expressed in standard deviations. Values of 0.90 or greater are considered large effect sizes, and, as such, are likely to be replicable in other studies. All data were analyzed using Prism 4.0b software (GraphPad Software, Inc., San Diego, CA, USA) and SPSS Statistics (IBM, Armonk, New York, USA) and are expressed as Mean \pm SEM. *A priori* significance threshold was set at $\alpha = .05$. Thus, p -values less than .05 are considered statistically significant.

Hypotheses

For the PND3 experiments, I hypothesized that CAF paired with an SAD would cause more apoptotic neurotoxicity than either CAF or SAD alone. Specifically, animals exposed to 80 mg/kg CAF and 6 mg/kg midazolam, 40 mg/kg ketamine, or 40 $\mu\text{g}/\text{kg}$ fentanyl would have higher mean density counts of apoptotic cells per mm^3 than animals exposed to CAF, midazolam, ketamine, or fentanyl alone.

For the multiple exposure experiments, I hypothesized that multiple exposures would cause less apoptotic neurotoxicity than a single exposure. Animals challenged on PND3 + PND6 with CAF + SAD were expected to have lower mean density counts of apoptotic cells per mm^3 than animals challenged only on PND6 with CAF + SAD. My

rationale was based on the possibility that the initial challenge on PND3 would kill the most vulnerable cells (Olney et al., 2002; Yuede et al., 2013), thus reducing the overall number of cells available for a second insult on PND6.

Results

Observations After Treatment

As expected, the chosen doses of 6 mg/kg midazolam, 40 mg/kg ketamine, and 40 µg/kg fentanyl were sub-sedative/anesthetic. None of the animals were immobilized or anesthetized and responded vigorously to tail and toe pinch after treatment. No animals displayed signs of respiratory impairment or skin discoloration, suggesting that animals were not hypoxic. Furthermore, after SAD treatment, animals were ambulatory and retained their righting reflex throughout the duration of all experiments.

Experiment 1: Apoptogenic Action of CAF + Midazolam at PND3

CAF was administered to PND3 mice at 80 mg/kg, and 6 mg/kg of midazolam was administered either alone or in combination with CAF. There was a significant overall effect of treatment, $F(3, 19) = 8.10$, $p = .001$, partial $\eta^2 = .561$. Fisher's post hoc analysis revealed that the CAF + midazolam group had higher mean density counts of apoptotic cells per mm³ than either the CAF or midazolam groups, $p = .01$ and $p = .002$, respectively (Figure 1). Further analyses yielded large effect sizes for both the CAF + midazolam versus CAF comparison (Cohen's $d = 2.72$) and the CAF + midazolam versus midazolam comparison (Cohen's $d = 2.56$). Taken together, the data suggest that CAF + midazolam co-exposure significantly increases apoptotic neurotoxicity compared to either drug alone.

Experiment 2: Apoptogenic Action of CAF + Ketamine at PND3

For experiment 2, I tested the pro-apoptotic action of 80 mg/kg CAF in conjunction with a 40 mg/kg dose of ketamine. There was a significant overall effect of treatment, $F(3, 17) = 4.71, p = .01$, partial $\eta^2 = .454$, and Fisher's post hoc revealed that the CAF + ketamine cocktail was more neurotoxic than either CAF or ketamine alone, $p = .003$ and $p = .013$, respectively (Figure 2). Effect sizes for both the CAF + ketamine versus CAF comparison (Cohen's $d = 1.75$) and the CAF + ketamine versus ketamine comparison (Cohen's $d = 1.48$) were large, suggesting that CAF + ketamine co-exposure reliably increases apoptosis in the PND3 brain.

Experiment 3: Apoptogenic Action of CAF + Fentanyl at PND3

Next, 80 mg/kg CAF was administered to PND3 mice, and 40 $\mu\text{g}/\text{kg}$ of fentanyl was administered either alone or in combination with CAF. Congruent with the midazolam and ketamine studies, there was a significant effect of treatment, $F(3, 20) = 9.77, p < .001$, partial $\eta^2 = .594$. Specifically, CAF + fentanyl increased mean density of apoptotic cells per mm^3 compared to the CAF-only and fentanyl-only group as evaluated by Fisher's post hoc, $p = .018$ and $p < .001$, respectively (Figure 3). Effect size analysis yielded large effect sizes for both the CAF + fentanyl versus CAF comparison (Cohen's $d = 1.23$) and the CAF + fentanyl versus fentanyl comparison (Cohen's $d = 2.47$). Thus, CAF + fentanyl co-exposure significantly and reliably increases apoptotic neurotoxicity compared to either drug alone.

Composite Dataset of the Apoptogenic Action of CAF + SADs at PND3

To provide a general overview of the apoptogenicity of CAF + SAD co-exposure, I generated a composite dataset by combining density counts from all three experiments into four groups: Control, SAD, CAF, and CAF + SAD (Figure 4). The doses selected for each of the SADs (midazolam, ketamine, and fentanyl) were sub-sedative/anesthetic doses, and in this composite dataset, it is clear that the chosen doses were sub-neurotoxic as well. While there was a significant effect of treatment, $F(3, 66) = 16.84, p < .001$, partial $\eta^2 = .434$, the SAD group did not significantly differ from Control in mean density of apoptotic cells. However, combining CAF with an SAD produced a consistent increase in apoptotic neurotoxicity across all SADs tested, $p < .001$ and $p < .001$, respectively.

In Figure 5, the composite dataset has been adjusted by subtracting out the density count of the Control group, which represents the natural background rate of physiologic apoptosis. The remaining values reflect the expected amount of apoptosis caused solely by drug exposure. Exposure to SAD or CAF would be expected to add approximately 22 and 48 apoptotic cells per mm^3 , respectively, to the PND3 brain. The dashed line represents the number of apoptotic cells per mm^3 one would expect by combining CAF with SADs ($22 + 48 = 70$ additional apoptotic cells per mm^3). However, the mean number of apoptotic cells in the CAF + SAD group was much greater than predicted (326 profiles per mm^3). On average, CAF combined with SADs resulted in an additional 256 apoptotic profiles per mm^3 in the PND3 brain.

These data suggest that CAF + SAD co-exposure significantly increases apoptotic neurotoxicity compared to either CAF or SAD alone and that the pro-apoptogenic action of CAF is supra-additive. Figures 6 through 9 are detailed light microscopy images of AC3 immunolabeling in brain regions important to cognition and behavior after PND3 CAF + SAD challenge.

Experiment 4: Apoptogenic Action of Single versus Multiple Exposures of CAF + Midazolam

In experiment 4, litters of mouse pups were injected with either vehicle, 6 mg/kg midazolam, 80 mg/kg CAF, or 80 mg/kg CAF + 6 mg/kg midazolam on PND3 and again on PND6. A separate set of litters received these treatments only on PND6, creating a 2 (Exposure: Single PND6 and Multiple PND3 + PND6) x 4 (Treatment: Control, Midazolam, CAF, CAF + Midazolam) factorial design. There was a significant main effect of Treatment, $F(3, 31) = 17.21, p < .001$, partial $\eta^2 = .625$. Collapsed across Exposure, Fisher's post hoc analysis revealed that CAF + midazolam significantly increased the mean density of apoptotic cells per mm^3 compared to CAF or midazolam alone, $p = .001$ and $p < .001$, respectively (Figure 10). The main effect of Exposure was not statistically significant, $F(1, 31) = .009, p = .923$, partial $\eta^2 = .000$. Thus, collapsed across Treatment, the PND6 brain exhibited a similar apoptotic response after multiple or single exposure. The Treatment x Exposure interaction was not significant, $F(3, 31) = .262, p = .853$, partial $\eta^2 = .025$, suggesting that the number of apoptotic cells caused by a given treatment was constant after single or multiple exposures.

Experiment 5: Apoptogenic Action of Single versus Multiple Exposures of CAF + Ketamine

Similar to experiment 4, experiment 5 yielded a 2 (Exposure: Single PND6 and Multiple PND3 + PND6) x 4 (Treatment: Control, 40 mg/kg Ketamine, 80 mg/kg CAF, 80 mg/kg CAF + 40 mg/kg Ketamine) factorial design. There was a significant main effect of Treatment, $F(3, 33) = 18.09, p < .001$, partial $\eta^2 = .622$, and Fisher's post hoc indicated that CAF + ketamine caused more neurotoxicity than either CAF or ketamine alone, $p = .001$ and $p < .001$, respectively. The main effect of Exposure was not statistically significant, $F(1, 33) = .665, p = .421$, partial $\eta^2 = .020$, nor was the Treatment x Exposure interaction, $F(3, 33) = .687, p = .5661$, partial $\eta^2 = .059$ (Figure 11).

Experiment 6: Apoptogenic Action of Single versus Multiple Exposures of CAF + Fentanyl

In experiment 6, 40 $\mu\text{g}/\text{kg}$ of fentanyl was administered alone or in combination with an 80 mg/kg dose of CAF on PND3. Animals received their respective treatment again on PND6 and were compared to a separate cohort that was treated only on PND6. The result was a 2 (Exposure: Single PND6 and Multiple PND3 + PND6) x 4 (Treatment: Control, Fentanyl, CAF, CAF + Fentanyl) factorial design. Similar to multiple exposure experiments with midazolam and ketamine, there was a significant main effect of Treatment, $F(3, 32) = 12.31, p < .001$, partial $\eta^2 = .536$. Collapsed across Exposure, Fisher's post hoc revealed that CAF + fentanyl significantly increased the mean density of apoptotic cells per mm^3 versus CAF or fentanyl alone, $p = .001$ and $p < .001$, respectively. The main effect of Exposure was not statistically significant, $F(1, 32) =$

.453, $p = .506$, partial $\eta^2 = .014$. Collapsed across Treatment, multiple exposures at PND3 + PND6 did not cause more or less neurotoxicity than a single exposure at PND6, suggesting the apoptotic response is relatively consistent after a single exposure or multiple exposures. The Treatment x Exposure interaction was not significant, $F(3, 32) = .703$, $p = .557$, partial $\eta^2 = .062$ (Figure 12).

Discussion

In a series of experiments, I report two important findings that contribute to the literature of the pro-apoptotic properties of caffeine (CAF) in developing brain. First, I demonstrated that the postnatal day 3 (PND3) mouse brain is highly vulnerable to apoptotic neurotoxicity of CAF and sedative/anesthetic (SAD) co-exposure. Compared to vehicle controls, neither 6 mg/kg midazolam, 40 mg/kg ketamine, nor 40 $\mu\text{g}/\text{kg}$ fentanyl were neurotoxic when administered alone. However, the addition of 80 mg/kg CAF to each of these agents caused a statistically significant, consistent increase in apoptotic neurotoxicity compared to either CAF or SAD alone.

Second, I demonstrated that the developing brain is as susceptible to CAF + SAD challenge after a single exposure as it is to multiple exposures. Mice treated with CAF + SAD at PND6 exhibited no difference in mean density of apoptotic cells compared to those treated at PND3 and again at PND6. Consistent with the findings in PND3 mice, the CAF + SAD cocktail was far more neurotoxic than either alone in PND6 animals, regardless of whether animals received a single exposure (PND6) or multiple exposures (PND3 + PND6). Since brain regions develop at different time points, premature infants exposed to semi-chronic or chronic regimens of CAF + SADs likely experience a

widespread, devastating loss of cells in neural substrates responsible for normal brain function.

I hypothesized that multiple exposures would cause less neurotoxicity than a single exposure. My rationale was that the initial insult on PND3 would kill the most vulnerable cells (Olney et al., 2002; Yuede et al., 2013), thus reducing the overall number of cells available for a second insult on PND6. Another consideration was that the surviving cells could resist a second insult by activating pro-survival signaling pathways and/or modulating the balance between pro- and anti-apoptotic proteins. Indeed, many NMDA antagonist or GABA-mimetic SADs harbor dose- and time-dependent neuroprotective properties by preserving adenosine triphosphate production, regulating intracellular calcium, and limiting neuroinflammation and astrocytosis (Berns, Seeberg, Schmidt, & Kerner, 2009; Harman et al., 2012; Rovnaghi, Garg, Hall, Bhutta, & Anand, 2008; Peng et al., 2014; Yan & Jiang, 2014; Zhan, Fahlman, & Bickler, 2006). These two outcomes are not mutually exclusive since it is plausible that the smaller pool of surviving cells could induce neuroprotective mechanisms, blunting the neurotoxic response from a second insult. My data argue against this possibility since the neurotoxic response was similar in magnitude between single and multiple exposure animals.

Given that there was no difference in neurotoxicity in PND6 brain after single versus multiple exposure, my results raise intriguing questions about the neurotoxic response of the developing brain to CAF + SAD challenge. The most logical possibility is that cells surviving the initial insult on PND3 are vulnerable to a second insult at PND6. In this scenario, surviving cells may have suppressed pro-survival signaling pathways and/or increased expression of pro-apoptotic proteins at PND6. Limited data

suggest that neonatal ketamine exposure upregulates NMDA receptor subunit NR1 protein expression, making NMDA receptors enriched with NR1 more susceptible to glutamate excitotoxicity (Slikker et al., 2007). Another study found that a cocktail of midazolam, nitrous oxide, and isoflurane caused a significant increase in reactive oxygen species that damage cells via DNA fragmentation, lipolysis, and proteolysis (Boscolo et al., 2013). In addition, ketamine exposure to neonatal mouse pups reduced levels of proteins in the pro-survival signaling pathways ERK1/2 and Akt (Straiko et al., 2009). Additionally, it is possible that the developing brain initiates a compensatory neurogenesis response after SAD challenge (Brambrink et al., 2012), and these newly generated cells may be susceptible to a later insult as they differentiate and form synapses. Yet, Stratmann et al. (2009) reported decreased neurogenesis in the dentate gyrus of neonatal rats after brief isoflurane exposure. This discrepancy may be attributable to species-specific differences in neuroregenerative capacity after SAD challenge or differences in choosing regions of interest and/or cell count methodology.

A third possibility involves the spatiotemporal development of the mammalian brain. Since brain development proceeds caudal-to-rostral and medial-to-lateral, neural substrates mature at different time points (Zhang et al., 2005). Furthermore, sub-nuclei in these regions are comprised of distinct populations of cells that also differ in rates of maturation (Zhang et al., 2005). Plausibly, a second exposure on PND6 may kill populations of cells that are beginning to mature around this time, making them vulnerable to CAF + SAD insult. Comprehensive experiments using protein analysis and histological methods in both non-human primate and rodents are needed to definitively answer these questions.

Interactions with Other NICU Drugs

Premature infants are often exposed to multiple drugs in the NICU for days, weeks, or months, and the neurotoxic consequences of these cocktails in the developing brain have not been studied in detail. For example, respiratory dysfunction is ubiquitous in preterm babies, and CAF is considered a “silver bullet” for treating apnea of prematurity, improving respiratory drive, and helping wean infants off of mechanical ventilation (Aranda et al., 2010; Henderson-Smart & Davis, 2003; Schmidt et al., 2006). Endogenous glucocorticoid signaling is essential for accelerating lung development and enhancing pulmonary surfactant production (Ballard & Ballard, 1995), and in neonatal intensive care settings, co-administration of CAF and glucocorticoids is common (Schmidt, Roberts, Millar, & Kirpalani, 2008).

However, postnatal exposure to glucocorticoids is associated with selective cerebellar stunting and permanent neuromotor and behavioral deficits (Parikh et al., 2007; Murphy et al., 2008; Tam et al., 2011). In a series of studies elucidating the mechanism of glucocorticoid-induced neuropathology, Noguchi and colleagues determined that a single dose of clinically-relevant glucocorticoids causes widespread apoptotic deletion of cerebellar neural progenitor cells destined to become mature granule neurons (Noguchi et al., 2008; Noguchi et al., 2011). Notably, animals treated with glucocorticoids had persistent neuromotor deficits well into adulthood (Maloney, Noguchi, Wozniak, Fowler, & Farber, 2011). Based on my studies, a compelling question is whether CAF has pro-apoptotic properties when combined with selective neuropathogens (glucocorticoid-induced toxicity in cerebellum) and/or other SADs that cause widespread neurotoxicity (sevoflurane, propofol, phenobarbital, nitrous oxide) in

the developing mammalian brain.

Pharmacological Neuroprotection for Developing Brain

Many SADs (including those used in the current experiments) and CAF are considered essential medicines for modern health care systems and are readily available in nearly every country (World Health Organization, 2014). Preterm infants often undergo multiple painful procedures and invasive surgeries during their stay in the NICU, and it would be both unethical and impossible to deliver medical care in the absence of sedatives, anesthetics, and analgesics. Decades of research support the beneficial effects of CAF as a safe, proven respiratory stimulant with few unwanted side effects. In neonatal intensive care settings, CAF and SADs likely have saved the lives of many preterm babies that would have otherwise died. Yet, CAF co-administered with SADs appears to trigger an aggressive, pro-apoptotic response in the immature mammalian brain that may lead to NDI in later life.

A practical solution to this conundrum is pharmacological neuroprotection, i.e., treating infants with neuroprotective agents during exposure to known apoptogens. Lithium is indicated as a treatment for major depressive disorder and bipolar disorder, but in recent years, it has been increasingly recognized as a potent neuroprotectant. Low dose lithium significantly attenuates developmental apoptosis caused by exposure to ethanol (Young, Straiko, Johnson, Creeley, & Olney, 2008; Zhong, Yang, Yao, & Lee, 2006), phencyclidine (Xia, Wang, Liu, Anastasio, & Johnson, 2008), and ketamine and propofol (Straiko et al., 2009). Furthermore, our lab recently reported that lithium provides dose-dependent neuroprotection against glucocorticoid-induced neurotoxicity in the developing cerebellum (Cabrera et al., 2014). While these data are promising, lithium

has a narrow therapeutic window, and concerns about the safety of lithium in the neonatal population have hampered its adoption in clinical settings.

Ideal pharmacological neuroprotectants should 1. Have a large therapeutic window to reduce the chance of unwanted side effects or overdose; 2. Not interfere with respiratory drive since nearly all preterm infants experience respiratory dysfunction and; 3. Be readily available in NICU settings. Dexmedetomidine, an α_2 adrenergic agonist, possesses these characteristics and is frequently used as a sedating agent in infants (Mason & Lerman, 2011). Importantly, dexmedetomidine significantly attenuates SAD apoptosis in developing rodent brain (Li et al., 2014, Sanders et al., 2009; Sanders et al., 2010). In a series of studies, our lab found that clinically-relevant doses of dexmedetomidine also protects the developing mouse cerebellum against glucocorticoid-induced apoptosis (O'Connor et al., submitted for publication). Thus, dexmedetomidine is an ideal candidate for future studies centered on the theme of pharmacological neuroprotection of the developing brain from CAF and SAD neurotoxicity.

Conclusion

Caffeine, midazolam, ketamine, and fentanyl have reputations of being safe and beneficial drugs in neonatal medicine, and premature infants often are co-administered high doses of SADs and CAF during their stay in the NICU to complete medical procedures and improve respiratory outcomes. However, clinicians and researchers are beginning to recognize that certain agents used in the NICU are neuropathogens that silently kill millions of brain cells. In contrast to the dogma of safety in the NICU, animal and clinical research suggest that exposure to therapeutic doses of SADs is

sufficient to cause long-term neuropathology and NDI in the developing brains of rodents, non-human primates, and human infants. In the present studies, I determined that CAF has pro-apoptotic properties in the PND3 and PND6 mouse brain when combined with sub-sedative/anesthetic doses of midazolam, ketamine, and fentanyl. Based on these results, it is recommended that clinicians exercise caution by limiting exposure to CAF and SAD to the minimal doses necessary to ensure the survival of babies born prematurely.

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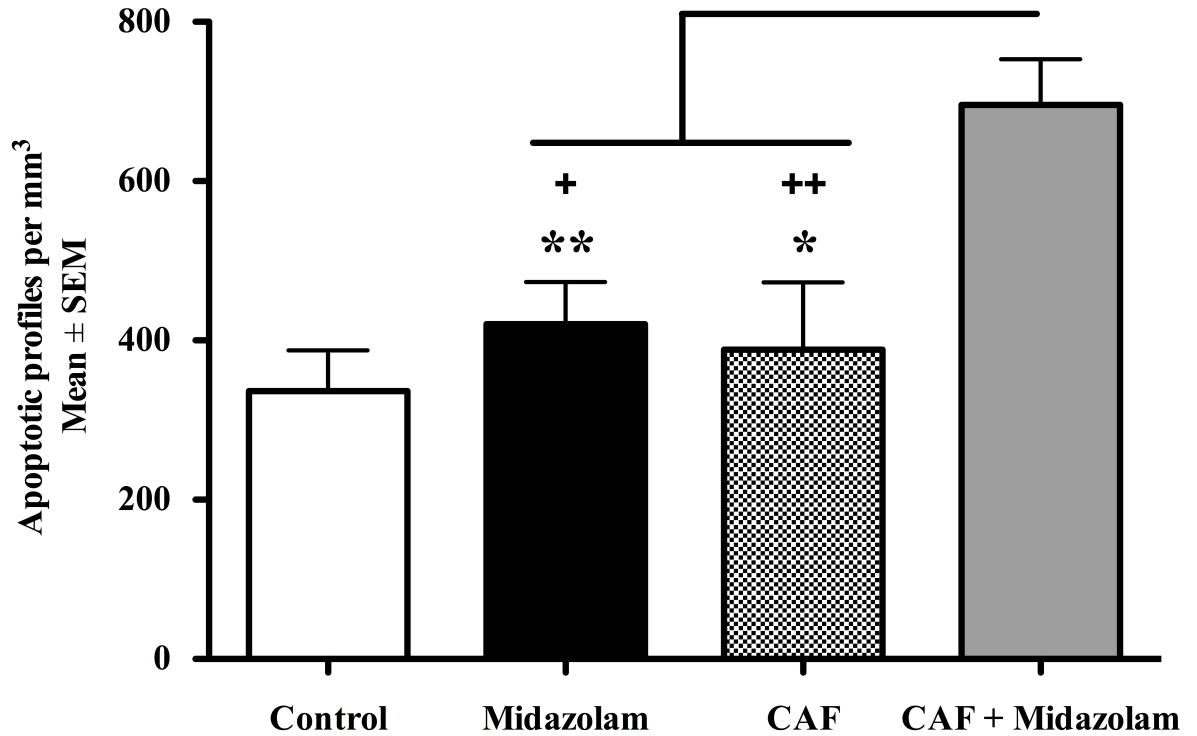
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Figures

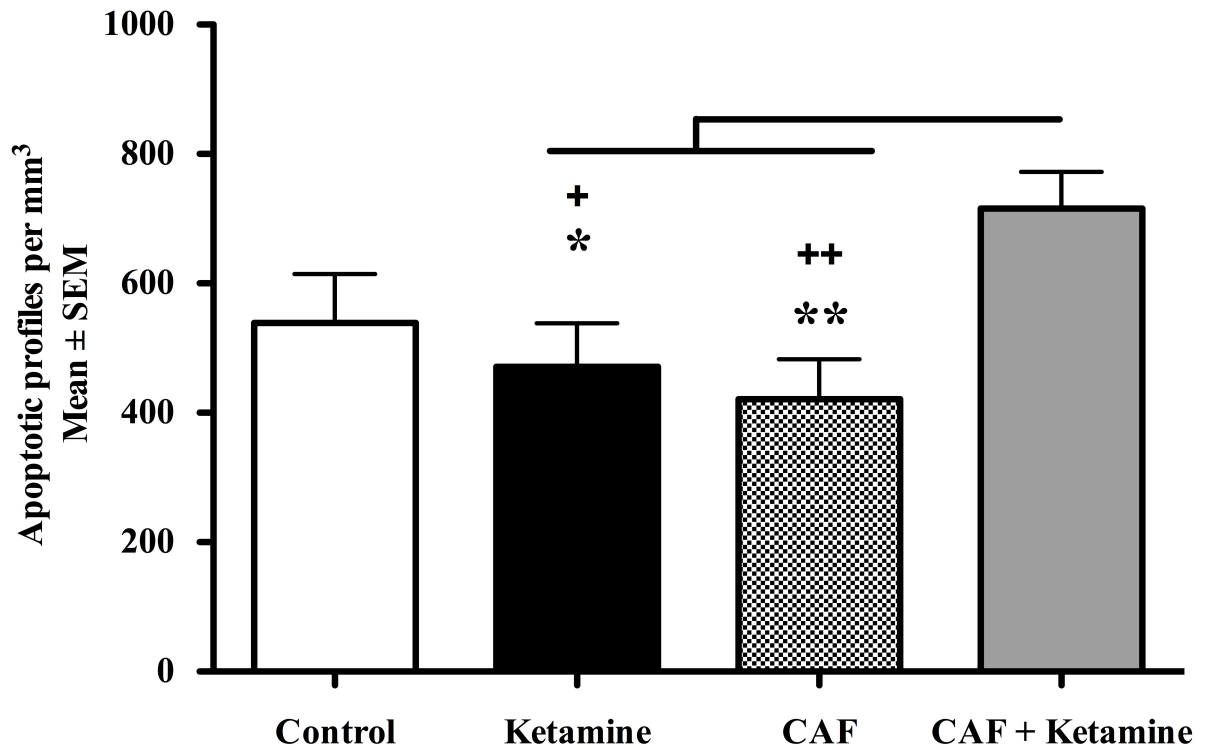


80 mg/kg CAF + 6 mg/kg Midazolam

Figure 1. Mean density counts of apoptotic profiles in the PND3 mouse brain after CAF + midazolam challenge. Apoptotic profiles were detected by activated caspase 3 (AC3) immunohistochemistry. Profile counts for the control group represent physiologic apoptosis, the background rate of natural cell death in the PND3 brain. A single exposure to 6 mg/kg midazolam or 80 mg/kg caffeine (CAF) caused no increase in apoptosis compared to controls. However, when CAF was combined with midazolam there was a statistically significant increase in apoptosis compared to either alone.

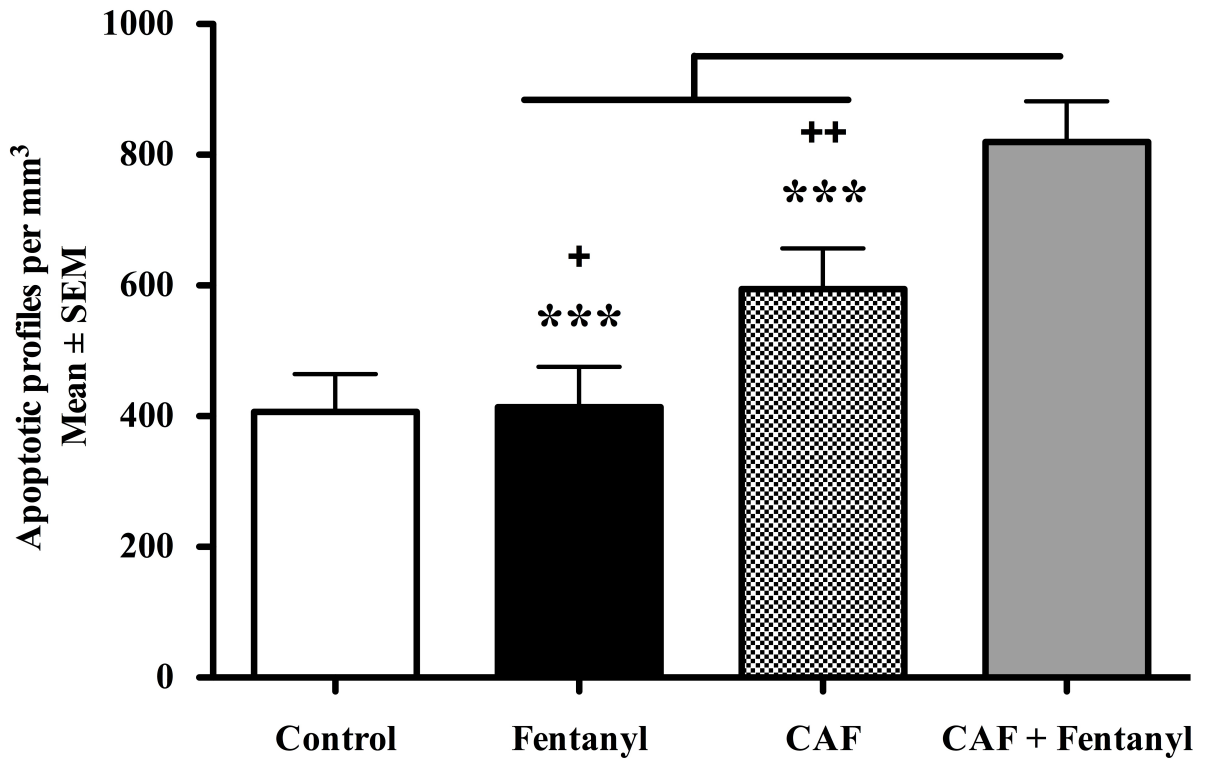
* $p < .05$ compared to CAF + Midazolam. ** $p < .01$ compared to CAF + Midazolam.

+Cohen's d CAF + Midazolam vs. Midazolam = 2.56. ++Cohen's d CAF + Midazolam vs. CAF = 2.72.



80 mg/kg CAF + 40mg/kg Ketamine

Figure 2. Mean density counts of apoptotic profiles in the PND3 mouse brain after CAF + ketamine challenge. A single exposure to 40 mg/kg ketamine or 80 mg/kg CAF caused no increase in apoptosis compared to controls. However, co-exposure to CAF + ketamine significantly increased AC3-positive cells compared to CAF or ketamine alone. * $p < .05$ compared to CAF + Ketamine. ** $p < .01$ compared to CAF + Ketamine. ⁺Cohen's d CAF + Ketamine vs. Ketamine = 1.45. ⁺⁺Cohen's d CAF + Ketamine vs. CAF = 1.75.

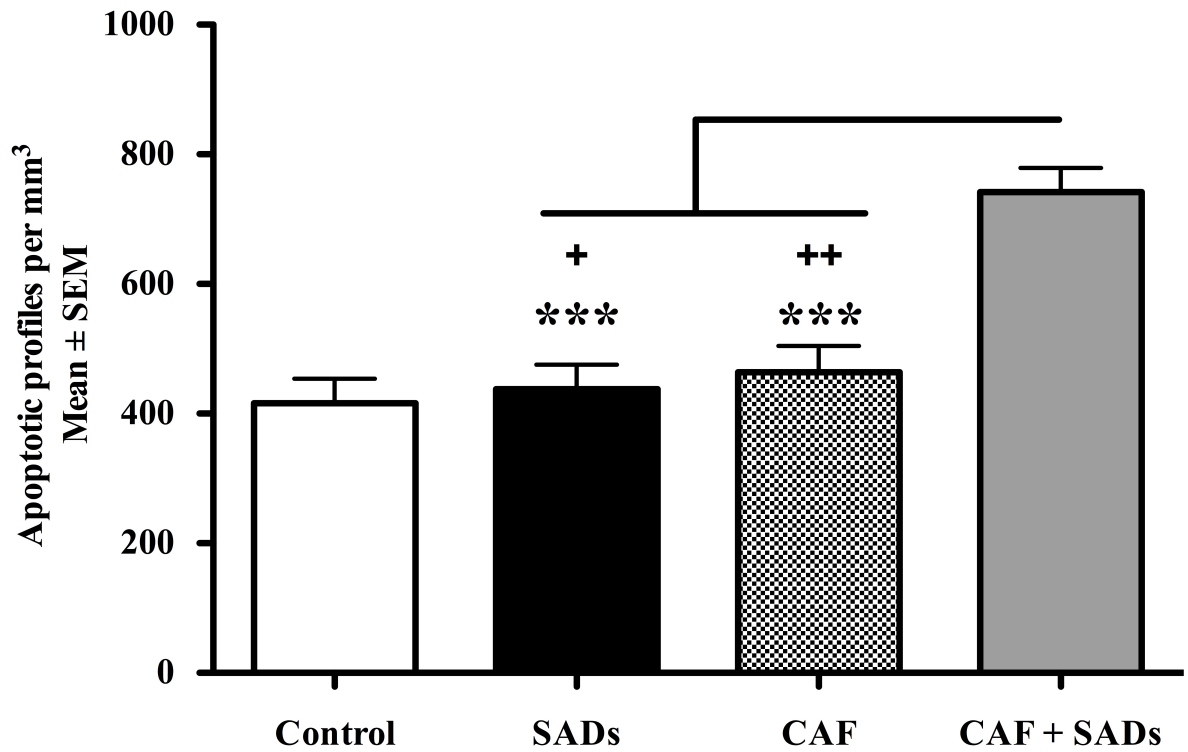


80 mg/kg CAF + 40 µg/kg Fentanyl

Figure 3. Apoptogenicity of CAF alone or in combination with fentanyl in PND3 mouse brain. AC3 immunolabeling revealed that a single exposure to 40 µg/kg fentanyl or 80 mg/kg CAF did not increase apoptosis compared to controls. However, the CAF + fentanyl cocktail caused a statistically significant increase in apoptosis compared to either drug alone.

* $p < .05$ compared to CAF + Fentanyl. *** $p < .001$ compared to CAF + Fentanyl.

⁺Cohen's d CAF + Fentanyl vs. Fentanyl = 2.47. ⁺⁺Cohen's d CAF + Fentanyl vs. CAF = 1.23.

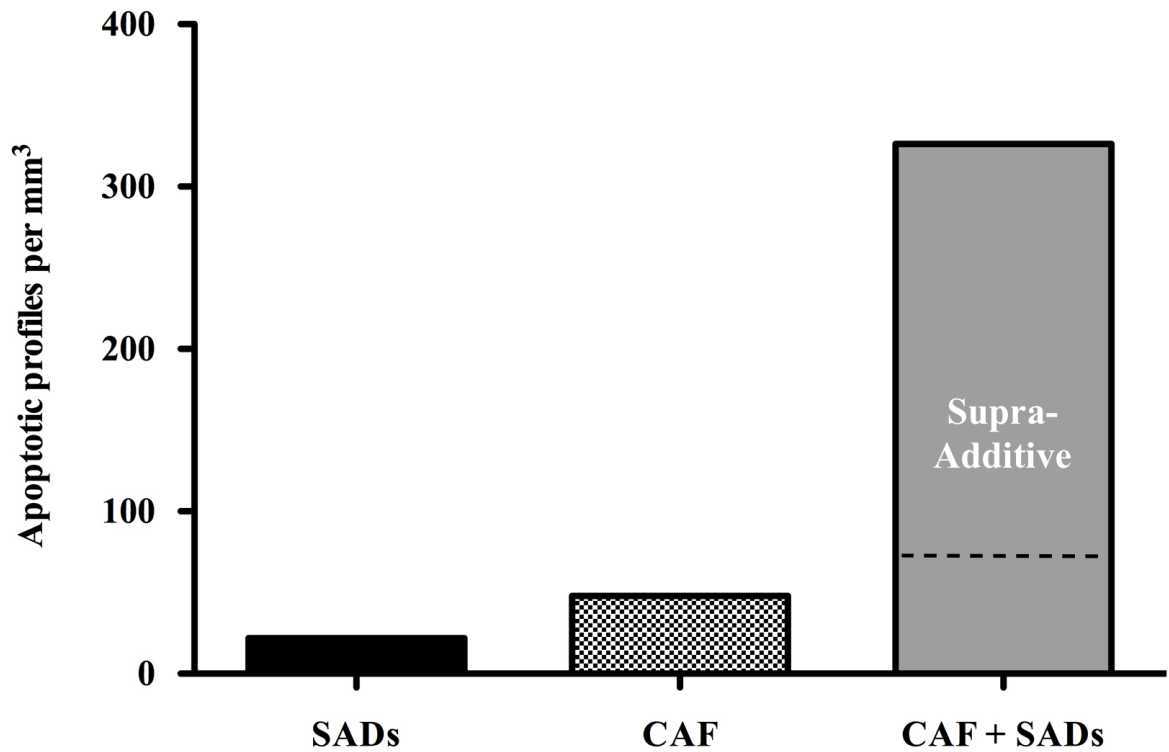


80 mg/kg CAF + SADs Combined

Figure 4. Overview of the apoptogenic action of CAF in combination with midazolam, ketamine, and fentanyl. To provide a general overview of the pro-apoptotic action CAF when combined with SADs, we generated a composite dataset of the density counts for all animals exposed to SADs, CAF, or CAF + SADs at PND3. In this composite dataset, the addition of CAF to all SADs tested caused significantly more apoptotic neurotoxicity than either CAF or SAD alone.

*** $p < .001$ compared to CAF + SADs.

⁺Cohen's d CAF + SADs vs. SADs = 2.10. ⁺⁺Cohen's d CAF + SADs vs. CAF = 1.66.



Cell Death Attributable to Drug Exposure

Figure 5. Composite dataset from Figure 4 adjusted to reflect cell death attributable to drug exposure. By subtracting out the Control value that represents the natural background rate of physiologic apoptosis, the remaining values reflect the amount of apoptotic cell death attributable to drug exposure. The dashed line represents the mean density of apoptotic cells that would be predicted by combining CAF with SADS. However, the actual mean density for the CAF + SAD group was much higher than predicted, suggesting that CAF has pro-apoptotic, supra-additive properties when combined with SADS.

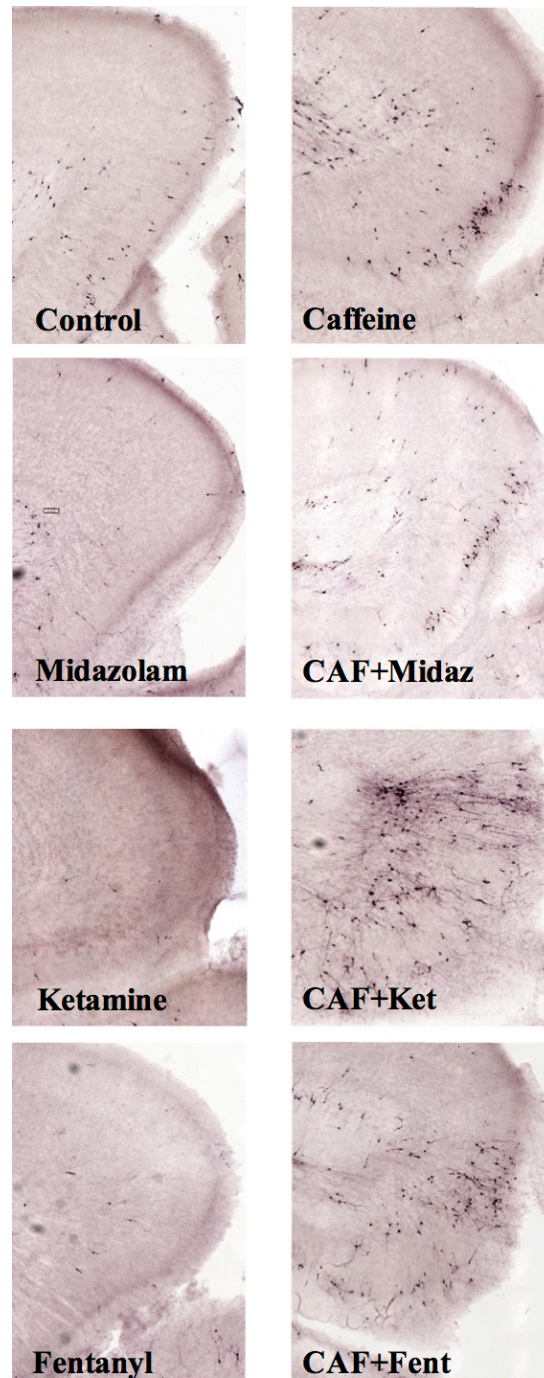


Figure 6. Representative images of CAF + SAD-induced apoptosis in the PND3 mouse retrosplenial cortex. Combining CAF with midazolam, ketamine, or fentanyl caused an increase in AC3-positive profiles in retrosplenial cortex. The apoptotic reaction involves cells in the superficial cortical layers as well as those in deeper cortical layers.

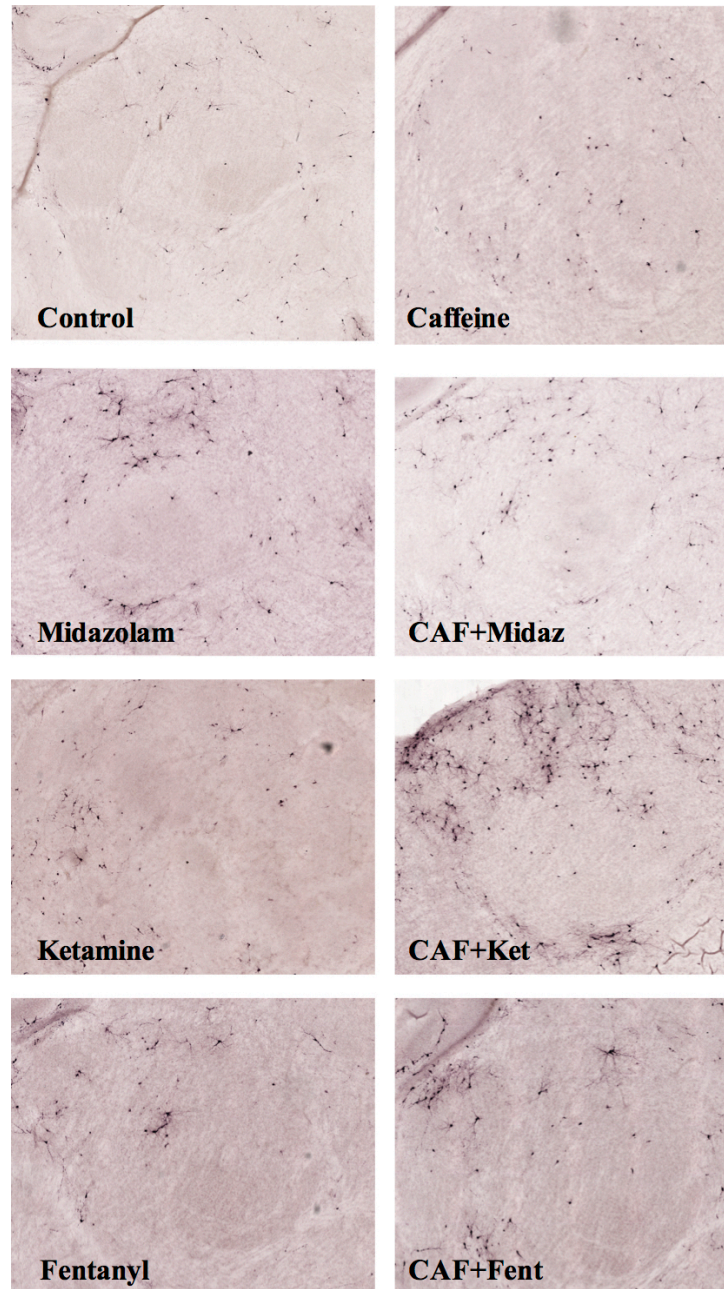


Figure 7. Detailed views of CAF + SAD apoptosis in PND3 mouse thalamus. At sub-sedative/anesthetic and sub-neurotoxic doses, midazolam, ketamine, and fentanyl did not cause a noticeable increase in apoptotic cells in thalamus. Only when combined with CAF did these agents alter thalamic apoptosis.

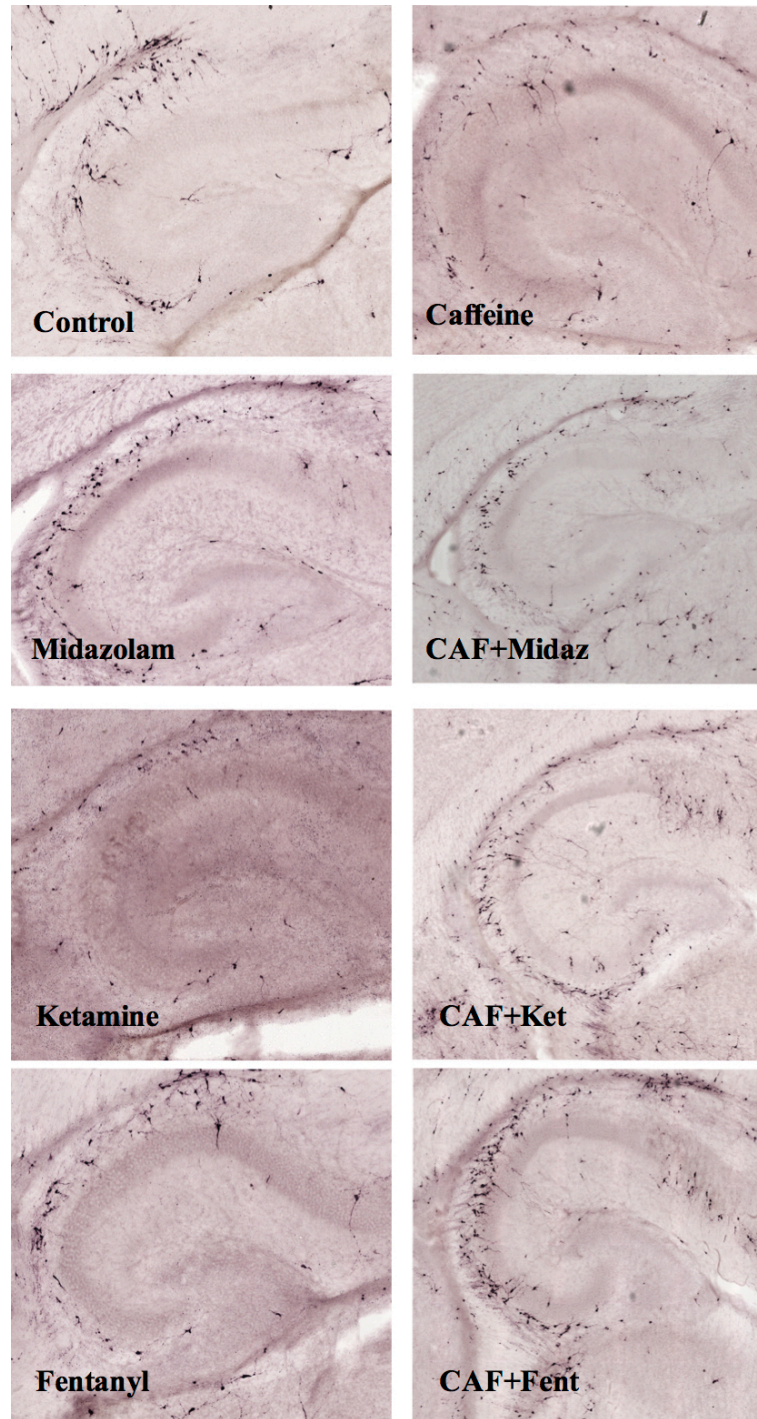


Figure 8. CAF + SAD-induced apoptosis in PND3 hippocampus. These detailed images reveal that pairing CAF with midazolam, ketamine, or fentanyl caused a marked increase in the density of apoptotic profiles in the hippocampal formation.

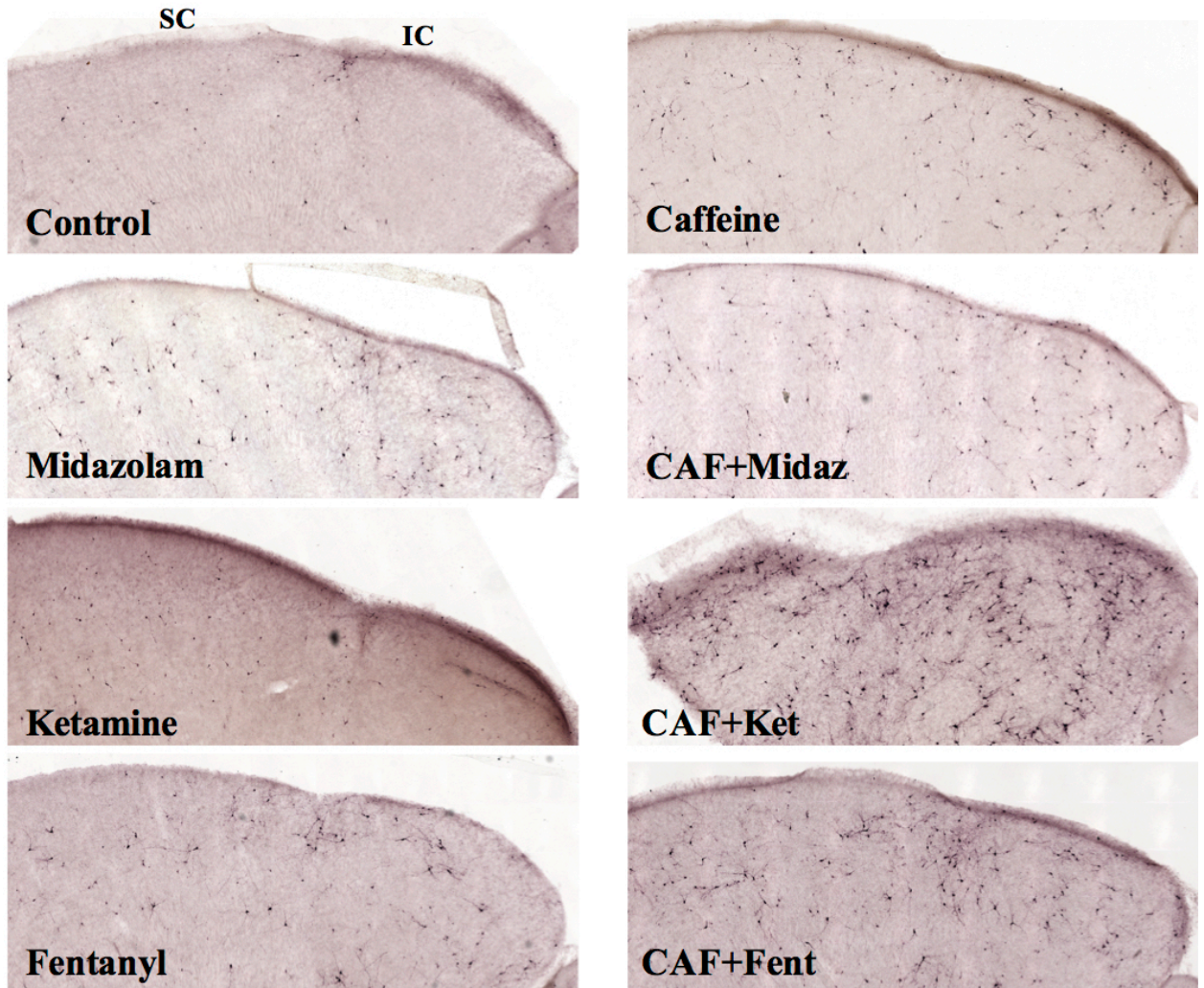


Figure 9. Detailed views of CAF + SAD-induced apoptosis in superior colliculus (SC) and inferior colliculus (IC). These images reveal that combining CAF with midazolam, ketamine, or fentanyl caused a marked increase in apoptosis in both SC and IC. Similar to retrosplenial cortex, thalamus, and hippocampus, the cells of animals treated with CAF plus an SAD are in an advanced stage of degeneration compared to CAF or SAD alone.

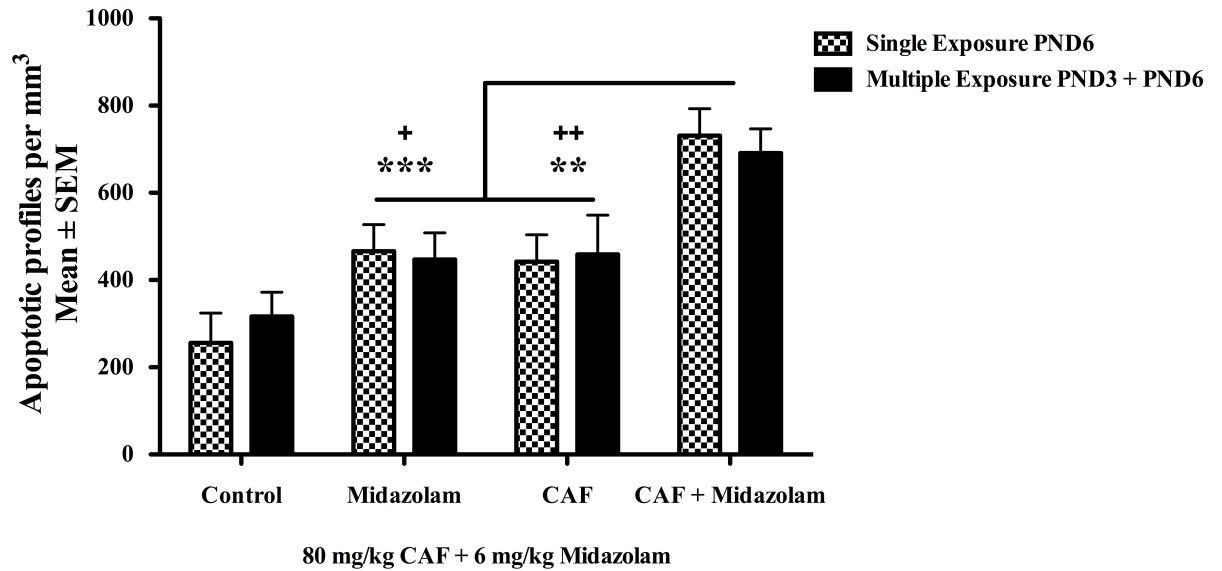


Figure 10. Apoptogenic action of CAF + Midazolam after single (PND6) versus multiple (PN3 + PND6) exposure. There was a significant main effect of Treatment. Collapsed across Exposure, mean density counts of apoptotic profiles were statistically significantly higher in the CAF + Midazolam group than the Midazolam or CAF groups. Our data suggest that the PND6 brain was equally vulnerable to single and multiple exposures of CAF + Midazolam.

*** $p < .001$ compared to CAF + Midazolam. ** $p < .01$ compared to CAF + Midazolam.

⁺Cohen's d CAF + Midazolam vs. Midazolam = 1.91. ⁺⁺Cohen's d CAF + Midazolam vs. CAF = 1.36.

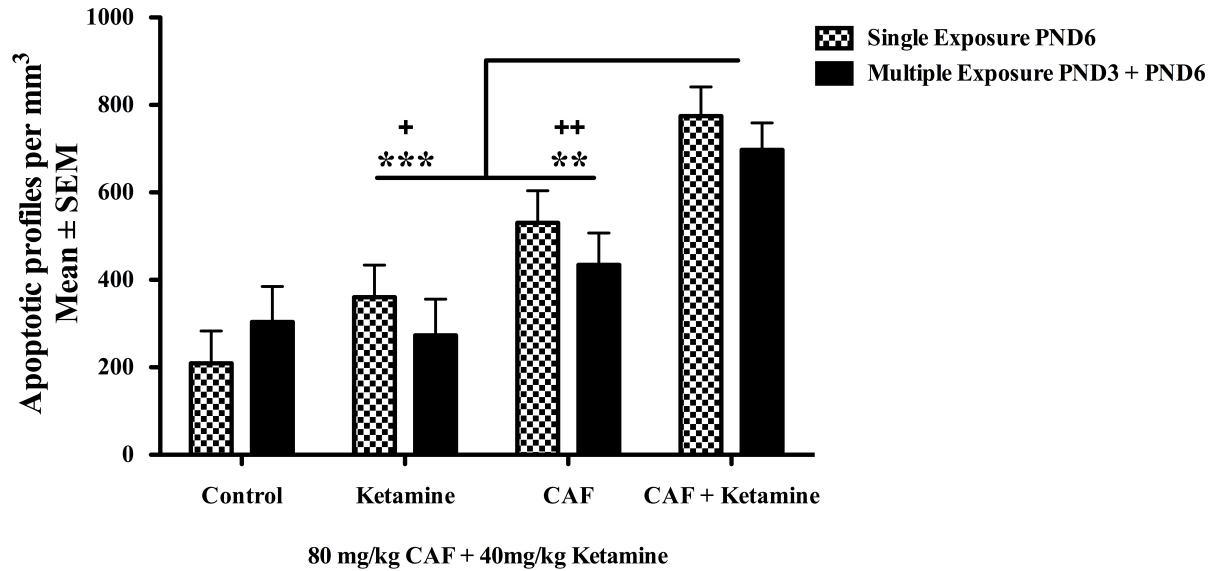


Figure 11. Apoptogenic action of CAF + Ketamine after single (PND6) versus multiple (PN3 + PND6) exposure. Collapsed across Exposure, mean density counts of apoptotic profiles were statistically significantly higher in the CAF + Ketamine group than the Ketamine or CAF groups. The data suggest that the PND6 brain was as susceptible to a single exposure of CAF + Ketamine as it was to multiple exposures. *** $p < .001$ compared to CAF + Ketamine. ** $p < .01$ compared to CAF + Ketamine. ⁺Cohen's d CAF + Ketamine vs. Ketamine = 2.22. ⁺⁺Cohen's d CAF + Ketamine vs. CAF = 1.35.

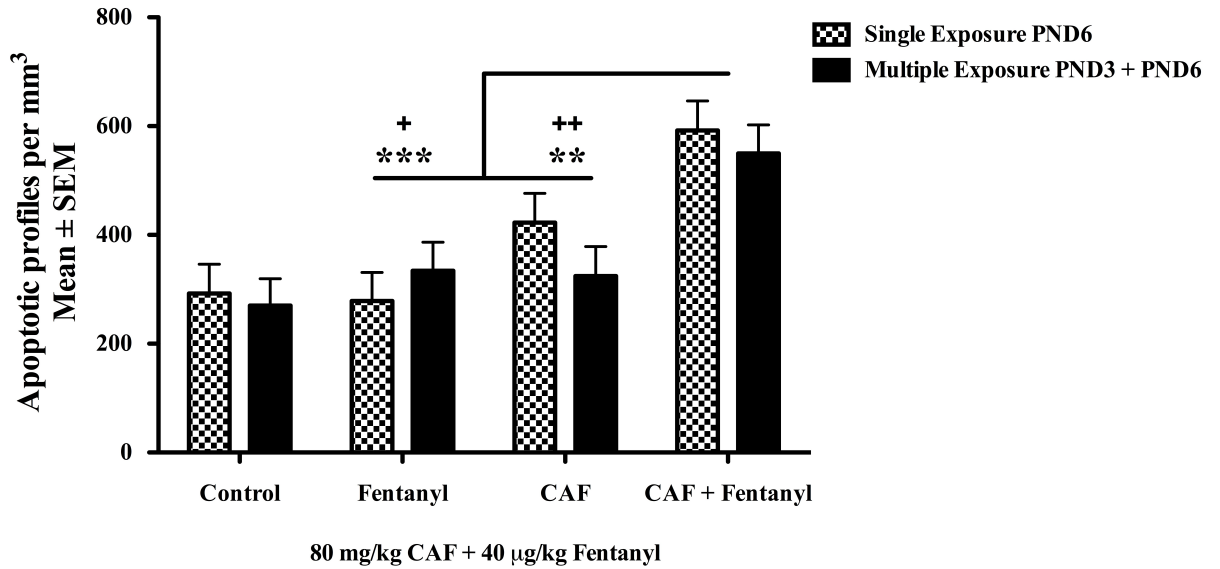


Figure 12. Apoptogenic action of CAF + Fentanyl after single (PND6) versus multiple (PN3 + PND6) exposure. Consistent with the midazolam and ketamine multiple exposure experiments, the significant main effect of Treatment and post hoc analysis suggests that the CAF + FENT cocktail caused more neurotoxicity than either Fentanyl or CAF alone.

*** $p < .001$ compared to CAF + Fentanyl. ** $p < .01$ compared to CAF + Fentanyl.

⁺Cohen's d CAF + Fentanyl vs. Fentanyl = 1.84. ⁺⁺Cohen's d CAF + Fentanyl vs. CAF = 1.49.