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Sedative and Neurotoxic Properties of Brexanolone Compared to Midazolam in the  
Developing Rodent Brain

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A Dissertation Submitted to The Graduate School at the University of Missouri - St.  
Louis in partial fulfillment of the requirements for the degree  
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Neuroscience

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### Abstract

The developing brain is susceptible to extensive neurotoxicity following exposure to sedative/anesthetic drugs (SADs). Every year hundreds of thousands of children around the world are exposed to SADs with no viable non-neurotoxic agents approved for clinical use. Allopregnanolone (AlloP) has well-established sedative effects in adults and neonates. AlloP and many SADs produce sedation/anesthesia through allosteric modulation of GABA<sub>A</sub> receptors, which is one of two principal mechanisms behind SAD-induced neurotoxicity. Evidence suggests AlloP has the unique capacity to regulate key apoptotic factors in adults and is widely involved with critical stages of neurodevelopment, indicating this neurosteroid might serve as a less neurotoxic sedative. Here, I showed the brexanolone (BRX) formulation of AlloP is more sedative than midazolam (MDZ). Lower doses of BRX had minimal impact on vital signs and produced no significant neurotoxic effect. Conversely, higher doses of BRX produced neurotoxicity, suggesting the apoptogenicity of BRX is dose dependent. Results for MDZ show far milder levels of sedation were obtainable until high doses. A significant neuroapoptotic response was induced at all sedative doses of MDZ. Prolonged light sedation similar to MDZ was obtained using BRX during continuous infusion. Although BRX appeared qualitatively less deleterious to heart and breath rate over time, 6-hour infusion of BRX induced a similar neurotoxic response compared to MDZ. These findings suggest low, sedative doses of BRX can be administered without gross impact to vital signs or neurotoxic consequences.

*Keywords:* allopregnanolone, brexanolone, sedation, midazolam, developmental neurotoxicity, neuroapoptosis

## **Sedative and Neurotoxic Properties of Brexanolone Compared to Midazolam in the Developing Rodent Brain**

### **Introduction**

A series of studies led us to what we now appreciate as widespread controversy over the use of sedative/anesthetic drugs (SADs) in pediatric and neonatal healthcare (Ikonomidou et al., 1999; Ikonomidou et al., 2000; Jevtovic-Todorovic et al., 2003; Olney et al., 2002). Experiments in rodents and non-human primates (NHP) continue to support the well-established concern that SADs produce extensive apoptosis throughout the developing brain. However, debate throughout the field of study has led some researchers to consider SAD-induced neurotoxicity an exaggerated concern (Hansen & Lönnqvist, 2016). Disagreement rests on a fine line between the different conclusions derived from animal models versus causal evidence linking human neonates exposed to SADs to long-term neurological and cognitive deficits (Davidson 2016).

In 2016 and 2017 the Food and Drug Administration (FDA) mandated label changes for many SADs. This warning cautioned patients and healthcare professionals on the use of SADs for multiple exposures or single exposures for more than 3 hours in children under 3 years of age and pregnant women (FDA 2018). The volume of preclinical data and correlative data from human studies speaks to the need for alternative compounds with less apoptogenicity but similar sedative and/or anesthetic efficacy compared to commonly used SADs (Lin et al., 2017; Walters & Paule, 2017). While there is a breadth of literature supporting long-term deficits after SAD administration in the developing brains of many non-human species (Maloney et al., 2018), conclusions derived from human studies are less convincing.

Largescale clinical trials have been conducted to help determine whether exposing the developing human brain to SADs creates short- and/or long-term neurobehavioral deficits. One such trial, the Pediatric Anesthesia Neurodevelopment Assessment (PANDA) study, included children exposed to a single regimen of volatile anesthetics before the age of 36 months compared to non-exposed siblings (Sun et al., 2016). Exposures ranging from 20min to 4hr (median 1.3hr) resulted in no significant changes in neurobehavioral domains compared to non-exposed siblings when measured in patients at 8 to 15 years of age (Sun et al., 2016).

Similar results were reported following the General Anesthesia Compared to Spinal Anesthesia (GAS) study. This randomized controlled trial compared a single exposure of less than 1hr sevoflurane on average anesthesia to awake-regional anesthesia in infants less than 1 year of age. No significant changes in neurodevelopmental outcomes were detected at 2 years of age using a cognitive composite score (Davidson et al., 2016). Additionally, deficits in intelligence quotient (IQ) and other behavioral indices were not detectable by 5 years of age, suggesting long-term neurobehavioral deficits may not occur following a single exposure to volatile anesthetics (McCann et al., 2019). Some data from the Mayo Anesthesia Safety in Kids (MASK) study similarly indicated a single exposure of anesthetics to children under 3 years of age produced no impairment in long-term IQ and various behavioral domains (Warner et al., 2018).

On the other hand, many cohort studies have shown results from single exposures to SADs are mixed. Children exposed to SADs before 3 years of age were at higher risk for impairments in language and abstract reasoning by age 10 (Ing et al., 2012). Exposure prior to the ages of 4 and 5 increased deficits in language skills, IQ, and academic

performance (Backeljauw et al., 2015; De Heer et al., 2017; Glatz et al., 2017). Single exposure to anesthesia before the age of 2 has been associated with lower recollection of associative information in children tested at ages 6 to 11 (Stratmann et al., 2014). Graham and colleagues (2016) showed no risk of long-term neurobehavioral deficits in children exposed to a single episode of anesthesia before the age of 2. This supports results from the PANDA, MASK, and GAS trials which investigated exposures during relatively early post-gestational periods. However, single exposures between ages 2 and 4 were related to deficits in communication and language domains (Graham et al., 2016). Others have similarly shown a single exposure to anesthetics before the age of 2 did not increase the probability of long-term harm while administration after 2 years of age did, suggesting earlier exposure to SADs might be less deleterious to long-term behavioral outcomes (O'Leary et al., 2016).

Results from well-controlled clinical trials are generally considered more reliable than cohort studies due to their experimental nature. However, it remains difficult to determine with certainty the full impact of a single SAD exposure given inconsistencies throughout the human literature. Nearly all clinical and cohort studies do not compare neurobehavioral outcomes of different drug regimens. The methods and drugs used for pre-, peri-, and post-operative procedures are often lumped together around frequency and/or duration of exposure within predetermined age groups. Likewise, no human experiments have detailed the long-term effects of combined SAD regimens compared pairwise to a single exposure of one anesthetic agent. Approximately 27% of children exposed to inhalant anesthetics in the PANDA study also received intravenous (IV) administration of propofol, thiopental, ketamine, or midazolam (MDZ). No indication

was given as to whether this group was statistically examined independent of other subjects (Sun et al., 2016). Preclinical studies have shown combinations of SADs produce an additive neurotoxic response compared to single administration, which can induce long-term neurobehavioral abnormalities (Fredriksson et al., 2007; Jevtovic-Todorovic et al., 2003; Young et al., 2005).

Largescale clinical trials have not yet investigated prolonged exposure to SADs. Many patients admitted to the neonatal intensive care unit (NICU), for example, experience reoccurring exposure to SADs and/or long-term maintenance of sedation that can span several days or even weeks (Hall & Shbarou, 2009). This is particularly concerning as preclinical evidence points to an intensified neurotoxic response with repeated and prolonged exposures (discussed in Maloney et al., 2019). Secondary outcomes from the MASK study indicate multiple exposures to SADs were associated with deficits in fine motor skills and cognitive processing speed (Warner et al., 2018). Similarly, the PANDA study found a weak association with abnormal internalizing behavior scores with longer SAD exposure (Sun et al., 2016). Several cohort studies also suggest multiple exposures to SADs may reflect an increased risk of learning disabilities, lower IQ, and language and cognition deficits (Flick et al., 2011; Glatz et al., 2017; Ing et al., 2012). Indeed, the risk for poorer learning outcomes was associated with greater durations of SAD exposure (Wilder et al., 2009).

Conflicting evidence derived from the human literature makes proving SAD-induced neurotoxicity in the developing human brain rather difficult. Still, preclinical evidence supports the concern that hundreds of thousands of neonates continue to be exposed to SADs every year. Medical care of children requires an ethical responsibility to

use SADs to ensure analgesia, anxiolysis, and survival of the newborn. The expanse of data from animal studies detailing the prevailing neurotoxic effect most SADs have on the developing rodent and NHP brain justifies continued research into whether SADs might be deleterious to the neurodevelopment of children. The need for alternative or adjunct treatments producing less neurotoxicity creates a central goal for future research.

## **Background**

### **Clinical Relevance of SAD-induced Neurotoxicity in the Developing Brain**

Although SADs employed in clinical practice have a wide range of mechanisms throughout the brain, the two mechanisms of action credited with developmental neurotoxicity involve *N-methyl-D-aspartate* (NMDA) receptor antagonism and  $\gamma$ -*aminobutyric acid* (GABA)<sub>A</sub> receptor agonism. Activities at NMDA receptors (NMDAR) and GABA<sub>A</sub> receptors (GABA<sub>A</sub>R) play key roles in maintaining a healthy trajectory of neurodevelopment. Disrupting the delicate balance of inhibition and excitation in the developing brain by either blocking NMDA activity or potentiating GABAergic signaling can lead to potent apoptotic responses far above normal levels observed during development.

Ikonomidou and colleagues (1999) were the first to detail how antagonism of the NMDA receptor leads to widespread neurotoxicity throughout the developing rodent brain. They found the non-competitive NMDAR antagonist dizocilpine (MK-801) produced a considerable neurotoxic effect in the PND7 rat brain. Cell death, although extensive throughout most of the brain, was greatest in the subiculum, laterodorsal thalamus, and layer II of the parietal, frontal, cingulate, and retrosplenial cortices. The neurotoxic effect was dose-dependent and detectable out to 24hr after administration.

NMDAR antagonists including, ketamine, phencyclidine, and carboxy-piperazine-4-yl-propyl-1-phosphonic acid (CPP) produced a near identical pattern of neurotoxicity.

Importantly, antagonists of dopamine, cholinergic muscarinic, and non-NMDA glutamate receptors, as well as  $\text{Ca}^{2+}$  ion channel blockers did not trigger a neurotoxic effect, indicating antagonism of NMDARs was the most likely cause of neuronal degeneration.

Follow-up research demonstrated ethanol, which has both NMDA and GABA<sub>A</sub> mimetic properties, produced neurotoxicity in the developing rat and mouse brain in a similar fashion as MK-801 (Ikonomidou et al., 2000; Olney et al., 2002). Their findings also revealed an additional pattern of neurodegeneration distinct from the various NMDAR antagonists, likely a consequence of ethanol's actions on GABA<sub>A</sub> (Ikonomidou et al., 1999). Administration of the benzodiazepines, diazepam and clonazepam, as well as barbiturates, pentobarbital and phenobarbital, induced a robust neurotoxic effect in layer IV of the parietal, frontal, and cingulate cortices, as opposed to more layer II specificity of MK-801. Other regions targeted more so by the neurotoxic effects of these GABA<sub>A</sub> agonists included the mediodorsal thalamus, septum, and CA1 region of the hippocampus (Ikonomidou et al., 2000). As such, it became clear drugs that function as antagonists or agonists of NMDAR and GABA<sub>A</sub>, respectively, are highly apoptogenic in the developing rodent brain.

Positive allosteric modulators of GABA<sub>A</sub> and NMDAR antagonists are commonly used in pediatric and obstetric medicine to induce sedation and/or anesthesia. To understand whether SADs also produced neurotoxicity, Jevtovic-Todorovic and colleagues (2003) employed various SADs common to clinical practice. They showed exposure to low doses of MDZ, a GABA<sub>A</sub> agonist, or  $\text{N}_2\text{O}$ , an NMDAR antagonist, did

not increase neuroapoptosis alone. On the other hand, isoflurane, which functions as both a GABA<sub>A</sub>R agonist and NMDAR antagonist, induced a dose-dependent increase in neuroapoptosis. The effect was most pronounced in layer II parietal cortex and the laterodorsal and anteroventral thalamic nuclei. A combination of MDZ with isoflurane potentiated the neurotoxic response. The most robust effect was observed with an initial injection of MDZ followed by 6hrs of isoflurane and N<sub>2</sub>O, which yielded a 20–60 fold increase in neuroapoptosis throughout various regions of the forebrain. Subsequent examination of long-term behavioral indices suggested the anesthetic cocktail induced learning and memory deficits detectable into adulthood. This was the first evidence suggesting SADs common to clinical practice might induce neurotoxicity in the developing brain with lasting neurobehavioral ramifications (Jevtovic-Todorovic et al., 2003).

Several rodent studies have since shown that a range of SADs commonly used in clinical practice can cause widespread neuroapoptosis throughout the developing brain and spinal cord (Walters & Paule, 2017). NMDA antagonists like ketamine and N<sub>2</sub>O, as well as GABA<sub>A</sub> positive allosteric modulators like propofol, diazepam, and MDZ, are regularly used in NICU and pediatric settings. Volatile inhalant anesthetics including sevoflurane, isoflurane, and desflurane that act on GABA<sub>A</sub> and NMDA receptors, are required for invasive surgical procedures. All these SADs trigger neuroapoptosis in the developing rodent brain and many have shown considerable potency even at sub-anesthetic doses (Cattano et al., 2008; Zhang et al., 2008).

Combining SADs is standard practice and preclinical studies have shown this can produce an additive effect that exacerbates neurotoxicity (Jevtovic-Todorovic et al., 2003;

Young et al., 2005). Additionally, caffeine is commonly used in the NICU to help prevent and recover apnea in premature infants. When co-administered with various SADs, caffeine can dramatically potentiate neurotoxicity in the developing rodent and NHP brain (Black et al., 2008; Cabrera et al., 2017; Noguchi et al., 2018; Yuede et al. 2013). These findings underline the fear that using SADs on humans during periods of neurodevelopment might cause enough neurotoxicity to produce permanent neurological and cognitive deficits.

The neurotoxic capabilities of SADs are dose- and age-dependent in the rodent brain and high levels of neuroapoptosis are observed during critical periods of myelinogenesis and synaptogenesis (Creeley & Olney, 2013; Maloney et al., 2018). This creates a window of vulnerability in which neurons are susceptible to SAD-induced apoptosis from approximately embryonic day (E) 19 to postnatal day (PND) 21 in the rodent brain (Olney et al., 2002; Dikranian et al., 2005). These ages roughly translate to humans as the second trimester through the first years of life.

A major concern regarding the translation of preclinical data to humans involves the possibility that SAD-induced neurotoxicity might not be legitimate or as detrimental to the developing human brain. Studies using NHP models have helped bridge this gap in translation because, unlike in rodents, vital signs and various physiological parameters can be accurately monitored during procedural models (Creeley & Olney, 2013).

Neuroapoptosis is evident in the neonatal and/or fetal NHP brain following exposure to ketamine, isoflurane, and propofol (Brambrink et al., 2010; Brambrink et al., 2012b; Creeley et al., 2013a; Creeley et al., 2014; Slikker et al., 2007; Zou et al., 2009; Zou et al., 2011). More recently, Noguchi and colleagues (2017) administered isoflurane

to neonatal macaques for 3 hours, a notably shorter duration of exposure compared to previous preclinical studies. Neuroapoptosis was measured to be 4-fold greater than air-only controls with most damage confined to the cortex, caudate, putamen, and thalamus (Noguchi et al., 2017). This suggests relatively short exposures to SADs might produce a troubling level of neurotoxicity.

An emerging finding is the nature of oligodendrocytes to undergo apoptosis following exposure to SADs. Susceptibility to oligoapoptosis is greatest during developmental periods involving myelinogenesis. Exposure to SADs may leave the brain vulnerable to deficiencies in white matter formation (Creeley & Olney, 2013; Maloney et al., 2018). Indeed, recent evidence has demonstrated white matter pathology, detected by noninvasive magnetic resonance imaging, in adult mice exposed to SADs at early periods of development (Noguchi et al., 2019). Although these data are not directly associated to oligoapoptosis, they emphasize the possible repercussions of SAD-induced neurotoxicity on oligodendrocyte proliferation and maturation and the formation of proper white matter circuits later in life.

As it currently stands, ethanol and various SADs, including isoflurane and propofol, result in extensive oligoapoptosis in the fetal and neonatal NHP brain, most of which is confined to subcortical white matter (Creeley et al., 2013a; Creeley et al., 2013b; Creeley et al., 2014; Noguchi et al., 2017; Brambrink et al., 2012a; Schenning et al., 2017). Vulnerability of oligodendrocytes persists beyond PND20-40 in the NHP brain (Schenning et al., 2017), which marks a developmental period that SAD-induced neuroapoptosis begins to decline. Rodent studies are less conclusive on the distribution and density of oligoapoptosis from SAD exposure. The window of vulnerability for

oligoapoptosis peaks around PND14, as opposed to the peak of susceptibility to neuroapoptosis at PND7 (Creeley & Olney, 2013; Ikonomidou et al., 1999).

### **Midazolam as a Commonly Used SAD**

Clinical sedation and anesthesia of children in the NICU is commonplace throughout healthcare institutes. Many emergency facilities that treat premature infants or very young children use a combination of drugs to ensure control of anxiety and pain during procedures. One of the most popular drug classes used in the NICU are benzodiazepines (BZD). For many years, diazepam was the main BZD used when treating children and adults due to its anxiolytic and sedative properties. However, its long half-life led to unintentional and prolonged sedation (Jacqz-Aigrain 1990) and has since been replaced by other BZDs.

Various SADs are employed in the NICU for sedation and analgesia but the most common of the BZDs administered is MDZ (Hall & Shbarou, 2009; Hall 2012). Neonates and preterm infants requiring mechanical ventilation are readily given MDZ to reduce agitation during the intubation process, as well as help synchronize respiratory function with the ventilator (Aranda et al., 2005). Additionally, MDZ has been shown to potentiate the analgesic effects of morphine (Hall & Shbarou, 2009), making it optimal for aiding pain management and sedation during pre- and post-operative procedures in children. The fast-acting capabilities of MDZ also make it practical for acute procedures in neonates where anxiolysis and sedation is required. MDZ is often favored for use in neonates due to the options of oral or nasal administration, avoiding the complications of IV or intramuscular administration in young patients.

Terminal elimination half-life of MDZ is an average of approximately 3 hours in human adults and around 2 hours in pediatric patients (FDA 2017; Pacifici 2014; Thummel et al., 2011). Doses range from 0.01–0.5mg/kg, depending on route of administration and patient profile. Intubated pediatric patients on continuous infusion of MDZ generally receive doses of 0.06–0.12mg/kg/hr (FDA 2017). Responsivity to MDZ depends mostly on dose and route of administration assuming the age and physical characteristics of patients are similar. However, there are limitations with modeling the various drug administration methods conducted in humans using neonatal rodents. The most reliable method for drug delivery in the neonatal mouse is acute injection and continuous infusions via the intraperitoneal (IP) route.

Sedative doses of MDZ are much higher in rodents than humans. In adult mice, the hypnotic ED<sub>50</sub> of MDZ to impair righting ability was previously shown to be approximately 43.5mg/kg (Ben-Shlomo et al., 2001). Mice administered 25mg/kg MDZ IP exhibited gait disturbances for approximately 30min, while 50mg/kg blunted righting reflex for 40min with defects lasting until the end of testing at 1 hour (Inada et al., 2004). Similarly, MDZ injected IP at 60mg/kg stunted righting ability for approximately 40min (Quinlan et al., 1998). Not surprisingly, 100mg/kg MDZ abolished righting reflex for roughly 1 hour (Dhir et al., 2013). No impact on righting ability has been shown for doses as low as 5mg/kg (Dhir et al., 2013; Besnier et al., 2018).

In neonatal rats, impaired latency to righting in PND10, but not PND3, pups were reported 15min after SC injection of 10mg/kg MDZ (Koch et al., 2008). Koyama and colleagues (2013) demonstrated 20mg/kg MDZ could decrease latency to righting in PND4 and PND7 rats 15min and 15-30min after injection, respectively. MDZ at 9mg/kg

induced behaviors suggestive of mild sedation but no loss of righting reflex in PND7 mouse pups was concluded (Young et al., 2005). Doses as low as 6mg/kg were considered sub-sedative and produced no identifiable impacts on righting ability (Cabrera et al., 2017). Importantly, MDZ has not been reported to induce anesthesia in rodents and is, thus, considered a sedative drug.

Clinical situations requiring long-term sedation of days or even weeks often use MDZ or a combination of MDZ with other SADs and/or caffeine. Milder states of sedation and reduced agitation can be maintained using MDZ for prolonged exposures. However, tension surrounds the use of MDZ and other SADs in the NICU and pediatric setting as the risk of neurotoxicity and subsequent long-term consequences are still poorly understood. There is a surprising lack of preclinical literature investigating the sole neurotoxic effects of MDZ following clinically relevant sedative doses. Some studies have employed subsedative doses of MDZ when using single injections or a combination of MDZ, caffeine, and/or other SADs (Cabrera et al., 2017; Young et al., 2005).

Early preclinical work showed a single injection of subsedative doses of MDZ (3, 6, or 9 mg/kg) did not produce greater levels of neurotoxicity compared to controls. Interestingly, a potentiated neurotoxic effect was observed when these subsedative doses of MDZ were co-administered with six hours of isoflurane. The addition of N<sub>2</sub>O to MDZ and isoflurane led to an even more widespread apoptotic response (Jevtovic-Todorovic et al., 2003). Follow-up research supported a measurable increase in neuroapoptosis in the caudate/putamen and cortex after a single 9 mg/kg injection of MDZ (Young et al., 2005). Combining MDZ and ketamine led to a greater neurotoxic response compared to either drug alone, despite both drugs independently increasing apoptosis when compared

to controls (Young et al., 2005). More recent research found the combination of caffeine with subsedative doses of MDZ (6 mg/kg) substantially increased neurotoxicity (Cabrera et al., 2017).

One issue with using subsedative doses of SADs in preclinical work is that results extrapolate the neurotoxic effects of the drug outside a clinical context. MDZ is used for sedation and anxiolysis appropriate for a variety of medical procedures. Situations warranting co-administration of MDZ with other SADs, such as volatile anesthetics or propofol, use acute administration of MDZ for pre- and post-operative care. In some cases, this is followed by sustained long-term sedation using MDZ which has rarely been examined in the preclinical literature. Recent work has employed more clinical-oriented paradigms (Maloney et al., 2018), but additional quantitative studies are required to determine neurotoxic profiles of sedative doses of MDZ more accurately.

### **Can Allopregnanolone Function as a SAD?**

Allopregnanolone (AlloP) is a neurosteroid synthesized from its parent compound, progesterone (P4). The enzyme  $5\alpha$ -reductase ( $5\alpha$ -R) catalyzes the conversion of P4 to  $5\alpha$ -dihydroprogesterone, followed by the synthesis of AlloP from  $3\alpha$ -hydroxysteroid dehydrogenase. Many SADs, including MDZ, depress brain activity through positive allosteric modulation of  $GABA_A$ R. The sedative capabilities of AlloP are similar in this respect and have long been shown to induce anxiolytic, anticonvulsant, and sedative effects.

Sedation and anesthesia are produced across a range of mechanisms for each class of drug, usually by binding of a specific receptor binding site. For instance, BZDs such as MDZ bind to the BZD allosteric binding sites located on  $\alpha$  and  $\gamma$   $GABA_A$ R subunits.

Various isoforms of the  $\alpha$  subunit ( $\alpha 1$ , 2, 3, and 5) present high affinity to BZDs and determine sensitivity to GABA-induced conformational change of the GABA<sub>A</sub>R.

Receptors containing the  $\alpha 1$  subunit are thought to produce the sedative and amnestic properties of BDZs, while those containing the  $\alpha 2$  subunit regulate anxiolysis (Weir et al., 2017). GABA<sub>A</sub>R of these isoforms are also highly concentrated in the cortex, thalamus, cerebellum, and limbic system (Brohan & Goudra, 2017), all of which are highly susceptible to SAD-induced neurotoxicity.

Excess GABAergic activation can stimulate brain regions that promote sleep behavior or inhibit networks involved with arousal and wakefulness. Brain regions associated with sedation and anesthesia include ventrolateral preoptic and tuberomammillary regions of the hypothalamus, pyramidal projections of the thalamus and cortex, and monoaminergic neurons of the brainstem and basal forebrain (Heemings et al., 2019; Spiegel et al., 2017). Importantly, the response from any given SAD, i.e. loss of consciousness, sedation, anxiolysis, etc. is specific to the mechanism and target region of the drug.

Allopregnanolone is a potent positive allosteric modulator of GABA<sub>A</sub>R with mechanisms similar to other SADs. AlloP-induced GABA activation is restricted to particular GABA<sub>A</sub>R isoforms, including some of those also bound by BZDs such as MDZ (Belelli et al., 2020; Brohan & Goudra, 2017). Indeed, high concentrations of AlloP can induce GABA<sub>A</sub>R activation in the absence of GABA (Carver & Reddy, 2013). Receptor subtype is also important as neurosteroids have generally been accepted as having low binding affinity for GABA<sub>A</sub>R containing the  $\gamma$  subunit, but high affinity for those containing the  $\delta$  subunit (Brickley & Mody, 2012). On the other hand, recent data

using  $\delta$  and  $\gamma 2$  knock-ins suggests this is not the case. AlloP has little selectivity between subunits and mediates tonic inhibition, or the sustained activation of GABA<sub>A</sub>R via extracellular GABA, regardless of exogenous GABA (Lu et al., 2020). This is important considering tonic inhibition by GABA<sub>A</sub>R is likely critical to the sedative/anesthetic effects of many drugs.

Analogs of AlloP may also function as reliable SADs. For instance, the AlloP analog 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane-11,20-dione, alfaxalone, is used as a SAD in veterinary medicine and has been shown to induce anesthesia following single injection and continuous infusion procedures in rodents (Montana et al., 2018; Siriarchavatana et al., 2016; White et al., 2017). Other preclinical work has shown alfaxalone has a higher therapeutic index compared to propofol (Goodchild et al., 2015; Tesic et al., 2020). Recent human trials have also been conducted using Phaxan<sup>TM</sup>, a 7-sulfobutylether  $\beta$ -cyclodextrin formulation of alfaxalone, for acute anesthesia. These studies demonstrated anesthesia was obtainable with lesser impact to vital signs, faster cognitive recovery, and better pain management when compared to propofol (Goodchild et al., 2015; Goodchild et al., 2020; Monagle et al., 2015).

Activation of GABA<sub>A</sub>R similar to AlloP has been reported with another analog, 3 $\alpha$ -hydroxy-3 $\beta$ -methyl-5 $\alpha$ -pregnan-20-one, ganaxolone (Belelli et al., 2020). However, the sedative and/or anesthetic side-effects are often considered unfavorable since ganaxolone is generally studied as seizure medication, making research into its clinical application as a SAD sparse. With that said, preclinical evidence on the sedative properties of endogenous neurosteroids confirms AlloP action at the GABA<sub>A</sub>R is particularly unique among neuroactive steroids. The sedating effects of P4 are often

recognized as the GABA<sub>A</sub> activity of AlloP (Reddy & Apanites, 2005). Mice lacking the P4 receptor are still susceptible to sedation by AlloP, supporting the role of AlloP and not P4 in sedation (Reddy & Zeng, 2007). In addition, AlloP has been shown to facilitate propofol-induced GABA<sub>A</sub> signaling in neocortical neuron cultures (Drexler et al., 2016). Similar reports demonstrated the combination of AlloP or ganaxolone with MDZ or tiagabine, a GABA-reuptake inhibitor, potentiated tonic inhibition in dentate gyrus granule cells (Chuang & Reddy, 2020). Combination of AlloP with other GABAergic SADs might reduce overall required dose in a clinical setting and, thus, neurotoxic and physiological side effects.

Despite these findings, the current AlloP literature focuses almost exclusively on treatment of adults with most studies viewing AlloP's sedative properties as an adverse side effect. Research focusing on the clinical application of AlloP normally concentrates on its analgesic, anxiolytic, and anticonvulsant potential (Poisbeau et al., 2014; Rogawski et al., 2013; Schumacher et al., 2014). Sedation has been commonly reported with AlloP in adult humans and rodents using a variety administration routes. However, only a handful of studies have reported the use of AlloP in neonatal rodents with supplementary observations on its sedative effects (Dhir & Chopra, 2015; Mares et al., 2006).

Mares and colleagues (2006) studied the anticonvulsant properties of AlloP. Their adjunct measures of motor activity indicated doses of 10 and 20mg/kg AlloP prolonged the latency to righting in PND12 rats. Deficits in various motor behaviors were observed as dose increased with age at 20mg/kg for PND18, 30mg/kg for PND25, and 40mg/kg for adult rats. Movement behaviors were not recorded in PND7 rats, but doses of 5mg/kg decreased the onset of drug-induced seizures implying AlloP activity at the level of the

CNS. All groups received AlloP via the IP route (Mares et al., 2006). Additional observations proposed doses of 5 and 10mg/kg AlloP induced sedation in PND9 rats, but no behavioral tests were conducted to substantiate the findings (Dhir & Chopra, 2015). These results point to the ability of AlloP to induce sedation in neonatal and young rodents.

To my knowledge, no studies have investigated AlloP in a context intended for sedation/anesthesia in neonatal rodents. Defining practical doses of AlloP becomes, consequentially, quite difficult since no pharmacokinetic or pharmacodynamic data have been published using neonatal rodent models. Based on findings in humans and adult rodents, and the limited data presented in neonatal rodents, AlloP could function as a SAD. A particular question of interest is whether sedation in neonates using AlloP might have consequences different from other SADs.

### **Importance of Allopregnanolone in Neuroprotection and Neurodevelopment**

Understanding the neuroprotective role of AlloP in the adult brain has gained traction over the years but is still largely understudied, especially in the developing brain. Several lines of research have focused on P4, AlloP's parent compound, as a neuroprotectant (De Nicola et al., 2018; Guennoun et al., 2015; Schumacher et al., 2014). However, the neuroprotective properties of P4 in the adult brain could be partly explained by AlloP (Irwin & Brinton, 2014; Liang & Rasmusson, 2018; Sayeed et al., 2006; Singh & Su, 2013). Indeed, AlloP has shown promising neuroprotective capabilities in Alzheimer's disease, Parkinson's disease, Multiple Sclerosis, traumatic brain injury, and stroke, among others (see Irwin & Brinton, 2014 for a comprehensive review).

One principal protective mechanism is AlloP's activity at the mitochondria, regulating apoptosis signaling. Sayeed and colleagues (2009) showed that AlloP, but not P4, inhibited the  $\text{Ca}^{2+}$ -induced opening of the mitochondrial permeability transition pore, a channel required for the release of cytochrome c (cyt c) from the mitochondria to activate apoptosis. Treatment with AlloP decreased cyt c secretion from the mitochondria following experimental TBI and stroke in adult rats, restricting intrinsic apoptosis signaling (Sayeed et al., 2009). Cell culture data support this showing AlloP decreased cyt c release and prevented intrinsic apoptosis signaling by suppressing Bax protein translocation at the level of the mitochondria (Xilouri & Papazafiri, 2006). In addition to modifying apoptosis signaling, AlloP also contributes to regulating production of reactive oxygen species, stimulation of neurotrophin production, and induction of anti-inflammatory responses, all of which are processes impaired by SADs (Johnson et al., 2019; Walter & Paule, 2017).

Allopregnanolone's role in development is also of significant importance when considering its neuroprotective properties in adults. AlloP is involved with the proliferation of human and rodent neural progenitor cells and has been proposed to stimulate hippocampal neurogenesis during embryonic development (Wang et al., 2005; Wang et al., 2008). Additionally, AlloP-induced activation of the  $\text{GABA}_A\text{R}$  is essential to the proliferation, differentiation, and maturation of oligodendrocytes at early periods of development (Schumacher et al., 2014). Cellular activation and gene regulation of both oligodendrocytes and Schwann cells can be modified by AlloP at the  $\text{GABA}_A\text{R}$  (Faroni & Magnaghi, 2011). Experiments with organotypic slice cultures from PND7 rat and mouse cerebellum demonstrated AlloP-induced  $\text{GABA}_A$  activation increased myelin

formation (Ghourmari et al., 2003), which may contribute to the formation of stable neuronal circuitry in the developing brain (Peper et al., 2011). Indeed, finasteride-induced deprivation of AlloP in the fetal guinea pig resulted in reduced myelin basic protein expression and elevated astrocyte activity (Kelleher et al., 2011). Administration of AlloP to rats during the first postnatal week modified the formation of neural circuitry within the cerebral cortex, producing behavioral alterations lasting into adulthood (Grobin et al., 2006).

Taken together, the protective and proliferative effects of AlloP might reduce SAD-induced neurotoxicity in the developing brain. Current research highlights the importance of AlloP in fetal and neonatal neurodevelopment. A growing body of evidence suggests AlloP might prevent neurotoxicity in models of fetal hypoxia (Yawno et al., 2009), play an important role in the maturation of oligodendrocytes, and aid in the formation of neuronal circuitry. From these stems the need to investigate whether sedation by AlloP might suppress neurotoxicity in the developing rodent brain.

As such, the goal of the current study was to explore the possibility that AlloP could serve as a sedative drug with less neurotoxic effects when compared to MDZ. Two clinically relevant scenarios were modeled using single administration for short-term sedation and continuous infusion for prolonged light sedation. Each experiment focused on the sedative capabilities, impact to vital signs, and the neurotoxic consequences of both drugs.

## **Method**

### **Animals and Drugs**

#### *Animals*

Post-natal day seven (PND7) ICR albino mouse pups of both sexes were used for all experimental procedures. PND7 mice represent a developmental age similar to the third trimester human fetus, making this age ideal for measuring maximal susceptibility to SAD-induced neurotoxicity (Ikonomidou et al., 1999; Creeley & Olney, 2013). Dams and litters were routinely monitored in an animal facility on the Washington University Medical School Campus. The colony was kept on a 12:12 light/dark cycle with room temperature at 21°C, humidity at approximately 50%, and *ad libitum* access to standard lab chow and water. All experimental procedures were approved by the Washington University Animal Studies Committee.

### ***Midazolam***

Midazolam hydrochloride (Hospira Inc., Lake Forest, IL, Midazolam Injection, USP) is a short-acting BZD that functions as a central nervous system (CNS) depressant. As a GABA<sub>A</sub> agonist with a relatively short terminal half-life, MDZ produces rapid sedation followed by a quick recovery period. MDZ is useful for sedation or anesthesia in adult and pediatric populations and is often used as an adjunct medication in pre- or post-operative settings.

Doses of MDZ were chosen based on data obtained from adult mice and the limited pharmacodynamic evidence in neonatal rodents. For acute sedation in experiment 1, low and high doses were chosen as roughly 1/3 and 3 times that of MDZ's ED<sub>50</sub> for hypnotic effect in adults, 15mg/kg and 120mg/kg, respectively (Ben-Shlomo et al., 2001). Middle doses of 30mg/kg and 60mg/kg were founded on the premise that sedation would incrementally scale with each dose, given the findings from adults noted above. Doses for

bolus and infused drug for prolonged sedation in experiment 2 were guided by prior experiments and observations made in-lab (Noguchi et al., 2019).

### ***Brexanolone***

Brexanolone (BRX) is a specific formulation of allopregnanolone mixed with a sulfobutylether- $\beta$ -cyclodextrin (Captisol) and was recently approved by the FDA for treatment of post-partum depression (FDA 2019a; ZULRESSO™). BRX was made to the same specifications as previously described (Kanes et al., 2017; Meltzer-Brody et al., 2018). Briefly, ALLO was mixed with Captisol (250mg/ml) in sterile distilled water at a concentration of 5 mg/mL and left to spin on a magnetic stirrer overnight. The solution was fully dissolved by briefly heating to 60°C then cooling to room temperature. Guidelines for BRX, specifically SAGE-547, administration require IV infusion over prolonged periods. This method is not possible for extended light sedation in PND7 mice; thus, all procedures administered BRX using the IP route as single injection or continuous infusion.

Doses for experiment 1 were chosen using in-lab observations, as well as previous research on AlloP in neonatal rodents noted above. Importantly, anesthesia, or the total loss of consciousness and absence of pain reflex, was not obtainable with any dose of BRX injected IP. Thus, the objective was to choose doses that elicited various levels of sedation. Single IP injection doses ranged from low (10mg/kg) to high (80mg/kg), with doses above 100mg/kg often causing death. Intermediate doses of 20 and 40mg/kg BRX were chosen based on prior data in PND12 and adult rats (Mares et al., 2006). Toxicology data on BRX in adult rats reported variable sedation with 8–12mg/kg/hr continuous IV infusion following a single 20mg/kg IV bolus injection (FDA 2019b; FDA 2019c).

Although variability was reported, these preliminary reports of BRX were used alongside my observations in lab to determine sedative vs. non-sedative doses for experiment 2. As such, a bolus IP injection of 20mg/kg preceded 10mg/kg/hr continuous IP infusion of BRX.

## **Procedures**

### ***Single Injection Experiment***

Mouse pups (PND7) in experiment 1 were randomly assigned to one of five doses within the BRX or MDZ treatment groups:  $n = 4$  for apoptosis profiles,  $n \geq 10$  for sedation measures,  $n = 6$  for pulse oximetry. Each litter of PND7 mice was placed in one of two drug treatment groups to ensure all doses of that drug were sufficiently represented for every experiment replication. Mice in the BRX condition received a single IP injection of either captisol vehicle at 7.5 $\mu$ l/g or BRX at 10 mg/kg, 20 mg/kg, 40 mg/kg, or 80 mg/kg. Mice in the MDZ condition received a single IP injection of either saline vehicle at 7.5 $\mu$ l/g or MDZ at 15 mg/kg, 30 mg/kg, 60 mg/kg, or 120 mg/kg. To account for unknown effects the captisol cyclodextrin might have on physiological neuroapoptosis, the dose for captisol controls was chosen as the average injection volume of all BRX doses. Injection volumes for saline were matched to captisol. Saline is the standard vehicle used in developmental neurotoxicity studies and has not been shown to alter physiological neuroapoptosis. All experimental animals were kept in a temperature-controlled chamber at 30 $\pm$ 0.5 $^{\circ}$ C. Previous research shows body temperature of  $\sim$ 35 $^{\circ}$ C significantly increased neurotoxicity in the developing brain, while hypothermic temperatures of  $\sim$ 24 $^{\circ}$ C can be neuroprotective. Temperatures around 30 $^{\circ}$ C show no

changes in neurotoxicity (Creeley et al., 2008; Yuede et al., 2009). Mice used for histological analysis were euthanized six hours after injection.

### ***6-hour Continuous Infusion Experiment***

Experiment 2 employed a continuous infusion paradigm (Harvard Apparatus PHD2000 Programmable Pump) modeling a state of light sedation sustainable over a prolonged period of time. Mice were split into groups of captisol control, saline control, BRX at 10 mg/kg/hr, or MDZ at 15 mg/kg/hr for 6-hours:  $n \geq 5$  for apoptosis profiles,  $n = 6$  for sedation measures,  $n \geq 5$  for pulse oximetry. To control for differences in total volume infused, vehicle (captisol for BRX, saline for MDZ) was used to raise the infusion volume of each animal to match the greatest injection volume. This ensured mice of the same drug group were infused with an equal total volume of liquid while also receiving the correct dose per hour. Infusion rate was adjusted for the largest infusion volume per hour  $\times$  6hrs.

Bolus IP injections BRX (20 mg/kg) and MDZ (30 mg/kg) were administered approximately 5min before infusion to ensure the animal was in a relaxed state during the initial stages of the procedure. Because GABAergic drugs induce neuroapoptosis in the developing brain, bolus doses were chosen to maximize sedation while minimizing the anticipated neurotoxic effect. Mice were then secured to a platform in either supine or prone position, depending on the goals of the experiment (see below), and a needle inserted into the abdomen and secured. Control animals were given a single injection of vehicle equivalent to the volume of bolus (captisol at 4 $\mu$ l/g and saline at 6 $\mu$ l/g). All mice were kept in a 30 $\pm$ 0.5 $^{\circ}$ C temperature-controlled chamber. Mice used for histological analysis were euthanized 6 hours after bolus injection.

### *Sedation Rating Scale*

No standardized procedures currently exist for measuring sedation/anesthesia in neonatal mice. Most scales describing behavioral difference throughout the continuum of sedation or anesthesia are in adult rodents (Chuck et al., 2006; Salamone et al., 1996). Others using sedation in neonatal mice simply measure the latency of the animal to right itself once placed in the supine position without considering other behavioral features. The American Society of Anesthesiologists (ASA) recently updated clinical guidelines for defining sedation and analgesia in humans (Apfelbaum et al., 2018). Responsivity to various levels of sedation, as defined by the ASA, include: 1) Normal response to verbal stimulation during minimal sedation (anxiolysis), 2) Purposeful response to verbal or tactile stimulation during moderate sedation, 3) purposeful response after repeated or painful stimulation during deep sedation, and 4) unarousable, despite painful stimuli during general anesthesia. These parameters of sedation are used to help clinically define various levels of sedation and were used as scaffolding in constructing my sedation rating scale.

I also reviewed the Neonatal Pain, Agitation, and Sedation Scale (N-PASS) to help understand the unique characteristics of neonatal sedation required in the NICU. This scale has been widely validated throughout a variety of clinical situations requiring neonatal sedation/analgesia (Deindl et al., 2013; Hillman et al., 2015; Hummel et al., 2008; Hummel et al., 2010). The N-PASS monitors sedation and pain across five assessment criteria: crying irritability, behavior state, facial expression, extremities tone, and vital signs (heart rate, blood pressure, breath rate, and oxygen saturation). Parameters for behavior state and extremities tone were considered during the development of the

present sedation rating scale. Vital signs were also monitored throughout the experiments.

Sedation is a state of induced relaxation defined subjectively by a medical professional's interpretation of the patient's physiological and behavioral state. Thus, defining sedation becomes even more difficult in the neonatal rodent as technological limitations prevent comprehensive monitoring of vitals and behavior. Based on the literature, I have developed a behavior-based scale to measure various levels of sedation in neonatal mice (Table 1). This scale includes latency to fully right, which has been previously used as a measure of sedation level in neonatal rodents (Koch et al., 2008; Koyama et al., 2013). These levels of sedation are based on the time and intensity of struggle the animal engages in while attempting to right itself.

<i>Sedation Rank</i>	<i>Behavioral Profile for Sedation</i>
5	<i>Anesthesia</i> : Animals is fully anesthetized (total loss of consciousness); displays no voluntary movement or tail pinch reflex.
4	<i>Deep sedation</i> : Animal cannot right itself and shows little to no voluntary movement; tail pinch elicits abdominal fluttering and slight movement of extremities; spontaneous twitching present when left undisturbed.
3	<i>Moderate sedation</i> : Animal can fully right itself within 15 seconds or partially right itself with minimal signs of struggle by rolling to its side; tail pinch elicits abdominal fluttering, potential vocalizations, and slight movement of extremities; spontaneous twitching present when left undisturbed.
2	<i>Mild sedation</i> : Animal can fully right itself within 10 seconds and shows moderate signs of struggle; motor activity includes swaying, rolling, twitching of the legs and torso; tail pinch elicits vocalizations and jerking of the fore and hind limbs.
1	<i>Residual sedation</i> : Animal can fully right itself within 6 seconds and displays obvious signs of struggle to maneuver to prone position; tail pinch elicits vocalizations and jolting of the fore and hind limbs
0	<i>No sedation</i> : Animal is fully awake and can fully right itself within 2-3 seconds.

Table 1. Behavioral profiles for ranked level of sedation

Ranking the sedative impact of each drug and dose for experiment 1 began 30min after initial injection. Animals were placed in the supine position, followed by light pressure applied to the tail. Observations were then recorded for 20secs. Various behaviors were examined throughout this period, including limb and body movement, spontaneous twitching, and time and difficulty spent engaged in righting reflex. Observations were noted once every 30min for 3 hours and assessed using the behavioral criteria described in Table 1 to determine the level of sedation.

The extent of sedation was defined as: anesthesia (Rank 5; complete loss of consciousness defined as absence of pain reflex), deep sedation (Rank 4), moderate sedation (Rank 3; clinically relevant; Apfelbaum et al., 2018), minimal sedation (Rank 2; anxiolysis), residual sedation (Rank 1), and fully awake (Rank 0). Moderate sedation is targeted by clinicians as this produces the least physiological complications while easing the struggle of prolonged procedures that might elicit anxiety, agitation, or even pain (Apfelbaum et al., 2018).

### ***Movement Quantification***

Almost all software used to measure movement focuses on detailed tracking of an animal's motion over space and time. The objective of this procedure was to accurately measure episodes of basic movement of the mouse pup's limbs and head while secured to a platform during infusion. Righting reflex is a natural tendency of the animal to flip itself to the prone position when placed on its back. By securing the animal in the supine position, this procedure can measure the level of awareness vs. sedation a given drug produces while in an adverse position.

Because behavioral evaluation of sedation began 30min after injection for experiment 1, possible drug influences may have been missed during the first 30min of movement. As such, movement was quantified for the first 30min after injection using a separate cohort of animals for experiment 1. Additionally, behavioral evaluation of sedation is not possible for mice in experiment 2 using the sedation scale (Table 1). Movement was recorded until a near identical amount of movement was obtained in both drug conditions, which was approximately at 4 to 5 hours of infusion. Mice were secured to a platform in the supine position approximately 5min after injection and recorded for 5 hours using a widefield camera to determine the length of time to achieve equal sedation. Videos were cropped for each animal and converted to MP4 format at SD 480p resolution (Uniconverter, Wondershare Technology Co., Ltd). Movement was monitored using a motion detection filter on VLC media player (VideoLAN).

For the Windows platform, the VLC motion detection filter can be accessed in the VLC program from the Tools tab → Effects and Filters (Ctrl+E) → Video Effects → Advanced → Motion detect. Once the “Motion detect” box is checked, all videos played through VLC will display boxes, or “shapes”, at each point of motion. Points of motion are determined by a contrast between one object moving over space relative to a stationary background. This contrast creates an undeterminable quantity of shapes varying by the intensity and duration of the movement episode. To optimize detecting movement of a PND7 mouse pup, the background must be clearly distinguishable from the animal and video resolution low enough to avoid false-positive detections. An additional precaution includes adjusting ambient lighting to avoid glares or shadows that

change in size or shape during recording. Videos were of the same file type and resolution throughout all experiments.

To begin testing, the video file was opened in VLC media player and paused, rewound back to 00:00sec, and playback speed set to 2.0x. Motion detection output is displayed in the “Messages” box accessible via Ctrl+M or Tools tab → Messages. To begin output, “Verbosity” should be set to “2 (debug)” and the video quickly started. The message board will display output as “motiondetect debug: Counted  $X$  moving shapes” where  $X$  is the number of shapes detected at the time of movement;  $X = 0$  when no movement is detected. Output data was saved as a .log file and imported into Excel for further processing. Because motion data are outputted as the number of moving shapes detected at the time of sampling, and the goal of the present experiment was to monitor general motion, all episodes of movement were converted to binary data, 1=motion, 0=no motion.

In some cases, background motion, or sampling noise, can be detected when the animal is visibly not moving. Sampling noise is defined as continuous motion detections generated by slight changes in lighting, shadows, etc. All videos were examined for sampling noise in a blind manner. Those deemed as having persistent sampling noise underwent the following noise-reduction procedure. To account for false-positive detections, three 10sec clips were randomly chosen from the video in which the animal was viewed as not moving but sampling noise was apparent. Motion detected during these clips was averaged as the total number of shapes detected over the three 10sec clips multiplied by the duration of the video. These data were subtracted from the total number of motion detections as an estimate of the number of falsely detected shapes. This process

was conducted for one video per experimental animal because ambient factors producing noise, i.e. lighting, animal breathing, etc., did not change across video segments.

Data were calculated as the percentage of motion shapes detected relative to all samples taken. Sampling frequency is roughly equivalent to the video's framerate (60fps), calculated by dividing the video's total frames outputted by the length (min) of the video. The result is then divided by 60 (sec) and multiplied by playback speed to determine the number of frames analyzed per second. Each video was analyzed independently of any other and included the duration of that video as a single timepoint within the experimental design. As such, videos for experiment 2 were broken into five 1hr segments.

### ***Monitoring Vital Signs***

Both experiments monitored oxygen saturation (SpO<sub>2</sub>), heart rate (BPM), breath rate (BrPM), and pulse distention (PD) using the MouseOx Plus Pulse Oximeter (STARR Life Sciences). Litters used were different from those processed for histological analysis and sedation ranking. In experiment 1, mice were lightly restrained while a throat sensor was placed around the dorsal region of the neck, just behind the ears. Valid readings were established after a stabilized 5-10sec signal (1Hz) with no error codes present for all four physiological parameters. Pulse oximetry signals were then examined and the most stable consecutive 5sec were averaged and taken as the final values for that measurement. Readings for experiment 1 took place at baseline, 5min after injection, and once every 30min for 3 hours. Experiment 2 employed a similar methodology. Baseline measures were taken prior to infusion. Animals were then secured to the infusion apparatus in the

supine position followed by pulse oximetry recording once every 30min for 6 hours. Data processing was the same for experiments 1 and 2.

### *Activated Caspase-3 Immunohistochemistry*

Animals processed for histological analysis for experiments 1 and 2 were sacrificed 6 hours after bolus injection as previously done (Cabrera et al., 2017; Maloney et al., 2019; Noguchi et al. 2019; Yuede et al., 2013). Previous work shows apoptotic cell death in the CA1 region of the hippocampus, as well as parietal, occipital, cingulate, and retrosplenial cortices was sufficiently detectable by 8hr after exposure. However, neuronal apoptosis was also evident throughout the neocortex by 4hr. Peak signaling was observed 4hr (for C57BL/6 mice) and 6hr (for ICR mice) after exposure in the caudate nucleus and nucleus accumbens and 8–10hr in anterior thalamic nuclei (Olney et al., 2002). The transient window of neuronal apoptosis is detectable in individual neurons over a 2 to 3hr period and regions containing similar cell-types from 6 to 8hr after the onset of apoptosis. As such, sacrifice 6hr after exposure supplies the best approximation of neurotoxic response (Olney et al., 2002).

All animals were deeply anesthetized followed by transcardial perfusion with a solution of 0.1% heparin in 4% paraformaldehyde. Brains were extracted and post-fixed in 4% paraformaldehyde for seven days. Brains were hemisected, embedded in 3.5% agarose, and sliced on a vibratome (Leica Biosystems, Buffalo Grove, IL, VT1000S) at 75 $\mu$ m. Serial sectioning yielded approximately 6 sagittal sections per brain.

Staining of neurotoxicity was accomplished using activated caspase-3 (AC3) immunohistochemistry. Sections were quenched in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10min. Sections then underwent a series of 5min washes in 0.01M phosphate buffered saline

(PBS) and 0.1% Triton X-100 followed by 1 hour blocking with 1x bovine serum albumin in PBS. Tissue was then incubated in blocking solution and anti-rabbit AC3 (Cell Signaling, Danvers, MA, Cat#9661) at a 1:1000 dilution overnight. Sections were then washed with PBS, incubated in a biotinylated goat anti-rabbit antibody (Vector Labs, Burlingame, CA, Cat#BA-1000) at 1:200 dilution for 1 hour, washed again, and then incubated in avidin-biotin complex (Vectastain, ABC Elite Kit) for 1 hour in the dark. Sections were developed using Vector-labs Violet Immunoperoxidase (VIP) to label AC3 positive cells dark purple.

### ***Cell Density Quantification***

Six sagittal serial sections spanning medial to lateral cortical and subcortical brain regions were traced. Regions of interest included the striatum, pallidum, all cerebrocortices and midbrain structures, and thalamic and hypothalamic nuclei. Regions excluded from the analysis included those comprising the hindbrain/cerebellum and olfactory systems. My preliminary analysis indicated these regions displayed little variability between BRX and MDZ treatments. All slices were analyzed using a Pentium III PC connected to a Prior Optiscan motorized stage (ES103 XYZ system, Prior Scientific Inc., Rockland MA) mounted on a Nikon Labophot-2 microscope. Quantification of cells positively labeled with AC3 was conducted in a blinded manner by the optical fractionator method using a Microbrightfield Stereo Investigator system (Microbrightfield, Inc., Williston, VT, USA) to generate an estimated population of apoptotic cells (Cabrera et al., 2017; Maloney et al., 2018; Yuede et al., 2013). Profiles for density of AC3-positive cells per  $\text{mm}^3$  were calculated by dividing the estimated population of apoptotic cells by total volume of traced brain regions.

### Statistical Analyses

Statistical analyses were performed using SPSS statistics 26 and GraphPad Prism 8. Evaluation of behavioral indices demonstrating level of sedation for experiment 1 were examined per drug, BRX or MDZ, using a 5 (Dose: Captisol or Saline control, 10 or 15mg/kg, 20 or 30mg/kg, 40 or 60mg/kg, 80 or 120mg/kg)  $\times$  7 (Time: Baseline, 30min, 1hr, 1.5hr, 2hr, 2.5hr, 3hr) mixed rmANOVA. Pulse oximetry parameters were analyzed using the same dose groups in a 5 (Dose)  $\times$  8 (Time: Baseline, 5min, 30min, 1hr, 1.5hr, 2hr, 2.5hr, 3hr) mixed rmANOVA to determine the acute and prolonged consequences of single drug injections. For experiment 2, general motion was used to gauge level of sedation by means of a 2 (Drug: BRX, MDZ)  $\times$  5 (Time: 1hr, 2hr, 3hr, 4hr, 5hr) mixed rmANOVA. Physiological parameters monitored by pulse oximetry were similarly examined by rmANOVA in a 2 (Drug: BRX, MDZ)  $\times$  13 (Time: Baseline, 30min, 1hr, 1.5hr, 2hr, 2.5hr, 3hr, 3.5hr, 4hr, 4.5hr, 5hr, 5.5hr, 6hr) design. Simple main effects were used to examine between- and within-subject differences following statistically significant main effects and interactions of repeated measures analyses. Bonferroni adjustment was used in all rmANOVA designs, as well as Greenhouse-Geisser correction when violations of sphericity were present. Dose-response curves were calculated by 4-parameter nonlinear logistic regression as drug dose plotted against sedation or estimated population of apoptotic cells. Neurotoxicity profiles for experiment 1 and experiment 2 were analyzed using One-way ANOVA with main factors of Dose and Drug, respectively, on density profiles of apoptotic cells per mm<sup>3</sup>. Supplemental analyses analyzed by One-way ANOVA include: 1) dose effect of BRX and MDZ on movement, 2) mean density profiles of apoptotic cells per mm<sup>3</sup> for the composite vehicle dataset, and

3) apoptotic profiles of single injection compared to continuous infusion. Tukey's HSD post hoc analysis was used following statistically significant ANOVA results. Evaluation of equally sedative doses of BRX and MDZ were examined by independent sample t-test.  $p < .05$  was considered statistically significant for all analyses.

## Results

### Experiment 1

#### *Brexanolone Induces Moderate-to-deep Sedation*

Brexanolone was administered as a single IP injection across of a range of doses to examine its low-to-high sedative potential. Behavioral indices for gauging sedation are detailed in Table 1. There was a significant main effect of Time across doses,  $F(4.20, 201.72) = 379.630, p < .001, \text{partial } \eta^2 = .888$ , as well as a significant Dose  $\times$  Time interaction,  $F(16.81, 201.72) = 40.230, p < .001, \text{partial } \eta^2 = .770$ , which were further examined for simple main effects. Sedation levels were defined by the onset of sedative effects from the drug given just after baseline, followed by an observed decrease in sedation as the drug was metabolized. Lower doses will elicit a lesser sedative effect compared to higher doses regardless of the timepoint examined. Response to any given dose is tracked by a decline in behaviors representing sedation, bringing the animal closer to wakefulness (Figure 1). Simple main effects were examined to determine within- and between-subject differences of the interaction, and all groups showed significantly lower levels of sedation at baseline compared to 30min post-injection.

Within-subject analysis showed sedation with BRX at 10mg/kg was significantly different compared to nearly every timepoint,  $F(6, 43) = 78.803, p < .001, \text{partial } \eta^2 = .917$ , with the exception of 2.5hr to 3hr, as well as 1hr compared to 1.5hr post-injection.

Measurements at hour 3 were also not statistically different from baseline, indicating a steady return to wakefulness from near moderate levels of sedation. Animals treated with 20mg/kg BRX were significantly different between almost every timepoint,  $F(6, 43) = 109.639$ ,  $p < .001$ , partial  $\eta^2 = .939$ , aside from comparisons of 30min to 1hr and 2hr to 2.5hr post-injection. Of note is the intensity of initial sedation produced by 20mg/kg BRX, i.e. moderate-to-deep sedation, and the rapid decrease to a residual sedative effect by the end of 3 hours. The decline of BRX's sedative effects were also significant for doses of 40 mg/kg,  $F(6, 43) = 103.579$ ,  $p < .001$ , partial  $\eta^2 = .935$ , and 80mg/kg,  $F(6, 43) = 107.792$ ,  $p < .001$ , partial  $\eta^2 = .938$ , albeit mostly uninterrupted until later timepoints. Specifically, animals administered 40 and 80mg/kg BRX showed minimal decline in sedation from near-deep to deep sedative effects until the last hour or 30min, respectively,  $p < .05$  for all.

Between-subjects analysis showed a significant difference between dose groups over time,  $F(4, 48) = 94.742$ ,  $p < .001$ , partial  $\eta^2 = .888$ . No significance values for a between-subject analysis were produced for baseline measures as all animals were fully awake prior to injection. Additionally, animals in the control group were significantly less sedated than all other groups at every timepoint except measurements at 2.5hr and 3hr, in which the 10mg/kg BRX group was not significantly different.

Simple main effects showed a significant difference in sedation between doses at 30min post-injection,  $F(4, 48) = 109.923$ ,  $p < .001$ , partial  $\eta^2 = .902$ . Animals in the 10mg/kg BRX group were less sedated compared to all other drug groups 30min after injection,  $p < .05$  for all. Doses of 20, 40, and 80mg/kg were not different from each other at 30min post-injection, suggesting the sedative effects of BRX reach an upper limit by

20 to 40mg/kg. This is also reinforced by my observations that BRX was unable to produce anesthesia before becoming lethal to the animal, even at very high doses not reported here. Data from this initial timepoint shows BRX can induce moderate-to-deep levels of sedation within 30min post-injection without driving the animal into a full anesthetic plane. By 1hr post-injection, a near identical between-group difference was revealed,  $F(4, 48) = 92.474$ ,  $p < .001$ , partial  $\eta^2 = .885$ , as all changes in sedation level were similar to the prior timepoint excluding the 20mg/kg group, which fell into a significantly less sedated state compared to the 80mg/kg group,  $p = .034$ . Sedation induced by doses of 40 and 80mg/kg BRX went relatively unchanged until after 2hr post-injection. Continued recovery from sedation was found at 1.5hr,  $F(4, 48) = 76.415$ ,  $p < .001$ , partial  $\eta^2 = .864$ , and 2hr,  $F(4, 48) = 63.040$ ,  $p < .001$ , partial  $\eta^2 = .840$ , post-injection between all group comparisons excluding 40 and 80mg/kg BRX,  $p < .01$ . A trend toward wakefulness persisted for 10 and 20mg/kg treated animals by 2.5hr,  $F(4, 48) = 70.011$ ,  $p < .001$ , partial  $\eta^2 = .854$ , and 3hr,  $F(4, 48) = 56.499$ ,  $p < .001$ , partial  $\eta^2 = .825$ , post-injection. Animals in the 10mg/kg BRX group were no longer significantly different from non-sedated controls by 2.5hr and 3hr measurements. Those that received 20mg/kg more or less reached residual sedation and were no longer separate from 10mg/kg animals by the final observation,  $p = .520$ . Although trending toward wakefulness, animals in the 40 and 80mg/kg groups were still near moderate and deep levels of sedation by the final measure, thus, significantly more sedated when individually compared to all other dose groups,  $p < .001$ .

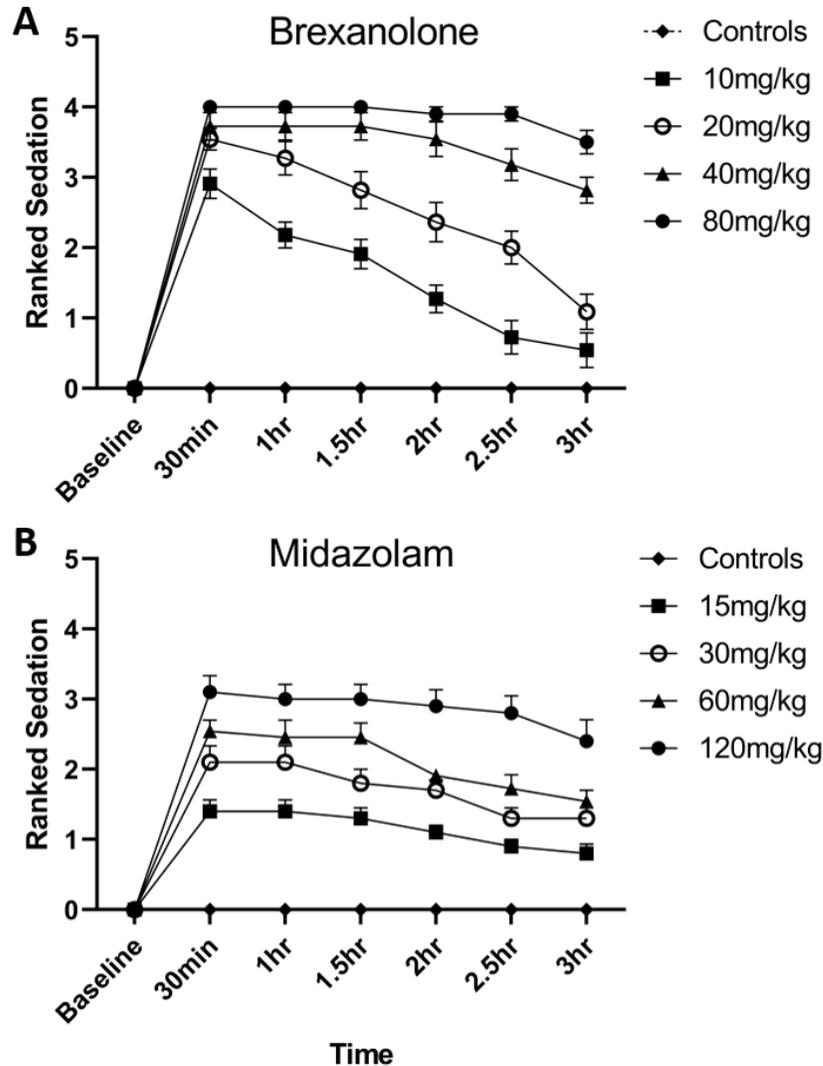


Figure 1. Behavioral observations used to determine level of sedation are detailed in Table 1. Sedation level was measured every 30min for 3hr. Drug was administered as a single IP injection. (A) captisol vehicle as control or with BRX at 10, 20, 40, and 80mg/kg; (B) saline vehicle as control or with MDZ at 15, 30, 60, and 120mg/kg.

### *Midazolam Induces Mild-to-moderate Sedation*

For MDZ treated animals, there was a significant main effect of Time across doses,  $F(3.539, 169.885) = 143.485, p < .001$ , partial  $\eta^2 = .749$ , revealing a change in sedation over the 3 hour sampling period (Figure 1). The analysis yielded a statistically significant Dose  $\times$  Time interaction,  $F(14.157, 169.885) = 40.230, p < .001$ , partial  $\eta^2 =$

.545. Within-subject analysis of simple main effects showed all groups displayed significantly higher levels of sedation at every timepoint post-injection compared to baseline.

Animals administered 15mg/kg MDZ were significantly more sedated at 30min and 2hr compared to 3hr post-injection,  $p < .05$  for all,  $F(6, 43) = 14.797$ ,  $p < .001$ , partial  $\eta^2 = .674$ , although beginning barely more sedated than the cutoff for residual sedation. Diminishing sedative effects were also apparent for 30mg/kg,  $F(6, 43) = 33.038$ ,  $p < .001$ , partial  $\eta^2 = .822$ , and 60mg/kg,  $F(6, 43) = 54.324$ ,  $p < .001$ , partial  $\eta^2 = .883$ . The level of sedation was significantly greater for both dose groups during 1.5hr to 2hr after drug administration compared to the last hour of behavioral analysis,  $p < .05$  for all. This suggests that, despite doubling the dose of MDZ from 30 to 60mg/kg, the efficacy of MDZ might be limited to sub-moderate levels of sedation. With that said, animals in the 120mg/kg MDZ group showed a significant decrease in sedation,  $F(6, 43) = 102.118$ ,  $p < .001$ , partial  $\eta^2 = .934$ , but only when sedation at 30min and 2.5hr post-injection were compared to the last behavioral observation at 3hr.

Between-subjects analysis showed a significant difference between dose groups over time,  $F(4, 48) = 77.320$ ,  $p < .001$ , partial  $\eta^2 = .866$ , of which simple main effects were used to determine specific differences. No significance values for the between-subject analysis were produced for baseline measures as all animals were fully awake prior to injection. Additionally, animals in the control group were significantly less sedated than all other groups at every timepoint,  $p < .05$  for all. The level of sedation at 30min,  $F(4, 48) = 51.735$ ,  $p < .001$ , 1hr,  $F(4, 48) = 40.122$ ,  $p < .001$ , partial  $\eta^2 = .770$ , and 1.5hr,  $F(4, 48) = 49.456$ ,  $p < .001$ , partial  $\eta^2 = .805$ , post-injection was significantly

different between doses. Animals treated with 15mg/kg MDZ showed milder levels of sedation compared to 60mg/kg and 120mg/kg groups,  $p < .001$ . Except for 60mg/kg MDZ compared to 30 and 120mg/kg, all remaining dose groups showed a spread in sedative effects from 30min to 1.5hr timepoints,  $p < .05$  for all. The trend toward wakefulness at 2hr post-injection was similar to prior timepoints with all dose groups other than 120mg/kg, stepping away from mild sedation and closer to wakefulness,  $F(4, 48) = 68.654$ ,  $p < .001$ , partial  $\eta^2 = .851$ . By 2.5hr,  $F(4, 48) = 43.551$ ,  $p < .001$ , partial  $\eta^2 = .784$ , and 3hr,  $F(4, 48) = 28.038$ ,  $p < .001$ , partial  $\eta^2 = .700$ , post-injection significant spreads in sedation were no longer present between animals treated with MDZ at 15 and 30mg/kg, as well as 30 and 60mg/kg. Animals in the 120mg/kg group were significantly more sedated than all other doses despite showing a stronger trend toward wakefulness.

#### ***Dose-response Curves for Sedative Effects of Brexanolone and Midazolam***

Maximum sedative effect of both drugs and all doses was achieved at the first behavioral observation. This assumes the maximal potency of each drug was reached within 30min of injection. The  $ED_{50}$  for sedation level produced by BRX was 4.7mg/kg (95% *CI*, 3.5-5.8; *R-squared* = .9891) while the  $ED_{50}$  for sedation with MDZ was 27.10mg/kg (95% *CI*, 19.03-105.2; *R-squared* = .9760), suggesting 50% of the maximum sedation obtainable from BRX requires a considerably lower dose relative to MDZ (Figure 2).

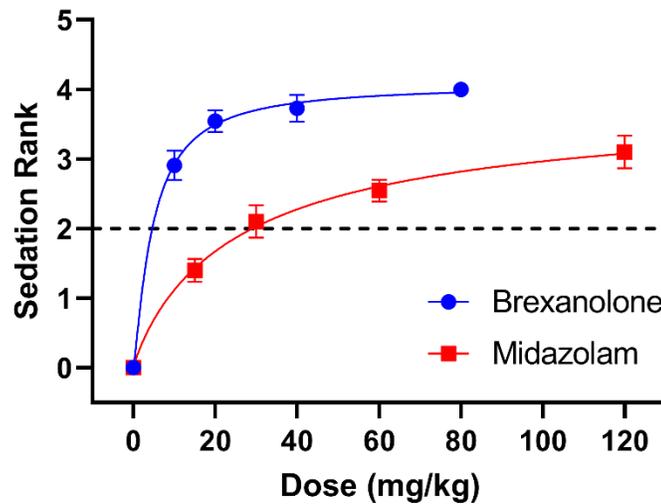


Figure 2. Dose-response curves for sedative effect of BRX and MDZ in PND7 mice 30min after single IP injection. Dashed line indicates  $ED_{50}$  for sedative effect of each drug. The  $ED_{50}$  for sedation of BRX was 4.7mg/kg to induce a mild level of sedation. The  $ED_{50}$  for sedation of MDZ was much higher at 27.10mg/kg, suggesting sizeable doses of MDZ were required to produce mild sedation. Neither drug surpassed the level of deep sedation (Rank 4), indicating both drugs were unable to generate anesthesia.

### *Brexanolone Weakens Certain Vital Signs at High Doses*

**Oxygen Saturation.** One of the most important variables to consider when administering a SAD in obstetric or pediatric medicine is ensuring the patient maintains a proper concentration of oxygen in arterial blood. Peripheral oxygen saturation (SpO<sub>2</sub>) is a measure of oxygen-bound hemoglobin that can be used to monitor whether a treatment produces hypoxemia. Results revealed a significant main effect of Time,  $F(3.984, 99.591) = 6.626, p < .001, \text{partial } \eta^2 = .210$ . Further analysis showed a significant Dose  $\times$  Time interaction,  $F(15.935, 99.591) = 40.230, p < .001, \text{partial } \eta^2 = .336$ , which was examined by simple main effects. Hypoxemia, or dangerously low levels of blood oxygenation, warrants immediate intervention when a patient drops below approximately 90% oxygen saturation. As such, animals administered 80mg/kg BRX were the focus of between- and within-subject differences. Although SpO<sub>2</sub> of animals administered

40mg/kg visibly dropped by 1hr post-injection, every dose other than 80mg/kg maintained SpO<sub>2</sub> of more than 95% (Figure 3A).

Within-subject analysis revealed the SpO<sub>2</sub> of animals in the 80mg/kg BRX group significantly changed over 3 hours,  $F(7, 19) = 30.192$ ,  $p < .001$ , partial  $\eta^2 = .918$ . Baseline SpO<sub>2</sub> was significantly greater compared to every other timepoint,  $p < .001$ . Within 5min, animals in the 80mg/kg group dropped to less than 90% SpO<sub>2</sub>, suggesting hypoxemia can be rapidly induced with high doses of BRX. Measurements at 30min point to a clear and potent effect on oxygen saturation, with 80mg/kg animals dipping below 85% SpO<sub>2</sub> and becoming statistically significantly less from all timepoints except 5min and 1hr. Recovery from hypoxemia is apparent following 30min post-injection as SpO<sub>2</sub> levels begin to recover with each consecutive timepoint. No significant differences in SpO<sub>2</sub> were detected between 1.5hr and 3hr timepoints. The average SpO<sub>2</sub> of the 80mg/kg group returns to approximately 90% by 1.5hr after injection and continues a slow climb back toward normal saturation.

Between-subject analysis showed a significant difference between dose groups,  $F(4, 25) = 22.732$ ,  $p < .001$ , partial  $\eta^2 = .784$ . Similar to the within-subject analysis, the focus of the between-subject analysis will be on animals administered 80mg/kg BRX. Simple main effects of the interaction revealed a significant between-subject difference at every timepoint post-injection,  $F(4, 25) = 4.684 - 13.321$ ,  $p < .01$ , partial  $\eta^2 = .428 - .681$ . Specifically, animals treated with 80mg/kg BRX showed significantly lower SpO<sub>2</sub> compared to all other groups at every timepoint,  $p < .05$ , with the exception of 80mg/kg compared to 40mg/kg BRX at 1.5hr post-injection. Interestingly, animals in the 40mg/kg group, and to a lesser extent 20mg/kg group, also show qualitative drops in SpO<sub>2</sub> at

30min post-injection followed by the gradual reinstatement of normal oxygen saturation. While neither were clinically or statistically significant, these data may help to better understand the pharmacodynamic profile of allopregnanolone in neonatal mice.

**Heart Rate.** An additional variable readily monitored during procedures employing SADs is heart rate. Previous data suggests the average heart rate of C57BL/6 mice at PND6/7 is  $582 \pm 45$  BPM (Zehendner et al., 2013). This is similar to the control data presented here with an average heart rate of approximately 522 BPM compiled across all timepoints. Results revealed a significant main effect of Time,  $F(2.804, 70.111) = 7.212, p < .001, \text{partial } \eta^2 = .224$ . Additionally, a significant Dose  $\times$  Time interaction,  $F(11.218, 70.11) = 2.724, p < .001, \text{partial } \eta^2 = .304$ , which were further analyzed by simple main effects.

Within-subject analysis revealed no significant changes in BPM in animals administered Captisol vehicle or 10mg/kg BRX,  $p = .731$  and  $p = .215$ , respectively. Similar to results of SpO<sub>2</sub>, the point in which most physiological ramifications of BRX occurred appears to be 1hr after injection. Animals exposed to 20mg/kg BRX displayed statistically significant changes to BPM over time,  $F(7, 19) = 7.520, p < .001, \text{partial } \eta^2 = .735$ . Heart rate was reduced at 1hr post-injection compared to 5min and 2–3hr timepoints,  $p < .01$  for all. Significant reductions in BPM were detected at 1hr and 1.5hr timepoints when compared to 2–3hr post-injection,  $p < .05$  for all, although these data point to a reinstatement of proper heart rate. By 2–3hrs post-injection, BPM became fairly stable showing significant differences only when compared at 30min – 1.5hr timepoints,  $p < .01$  for all.

Animals administered 40mg/kg BRX also showed statistically significant changes in BPM throughout the 3hr period,  $F(7, 19) = 6.034$ ,  $p < .001$ , partial  $\eta^2 = .690$ . No changes were detected until 1hr post-injection, which revealed lower BPM when compared to the 3hr timepoint,  $p = .015$ . From there, no differences were detected until comparing 1hr and 1.5hr post-injection to the 3hr timepoint,  $p < .05$ . Although these data show a similar effect as their 20mg/kg counterparts in Figure 3B, the recovery from BRX's impact to heart rate appeared less robust. Animals treated with 80mg/kg BRX showed the most pronounced response to the drug,  $F(7, 19) = 3.021$ ,  $p = .026$ , partial  $\eta^2 = .527$ , illustrated in Figure 3B by a drop in heart rate below 350 BPM. On the other hand, statistical evaluation of BRX's impact yielded no significant changes when comparing 5min–1hr timepoints. By 1.5hr and 2hr post-injection, significant drops in BPM were detected,  $p < .05$ , disappearing as the animals slowly recovered from the drug's effects.

Evaluation of between-subject effects revealed a significant main effect of Dose,  $F(4, 25) = 10.220$ ,  $p < .001$ , partial  $\eta^2 = .621$ . No between-subject differences were detected at baseline. Statistically significant changes were found at 5min post-injection,  $F(4, 25) = 3.289$ ,  $p = .027$ , partial  $\eta^2 = .345$ , indicating animals in the 10mg/kg group had higher BPM compared to those that received 80mg/kg BRX,  $p = .017$ . Significant reductions in BPM were also detected at 30min post-injection,  $F(4, 25) = 5.346$ ,  $p = .003$ , partial  $\eta^2 = .461$ , in the 20mg/kg group compared to controls,  $p = .042$ . Additionally, reduced BPM was shown at 30min and 1hr,  $F(4, 25) = 5.727$ ,  $p = .002$ , partial  $\eta^2 = .478$ , timepoints in 80mg/kg animals compared to control and 10mg/kg groups,  $p < .05$  for all. By 1.5hr post-injection,  $F(4, 25) = 15.283$ ,  $p < .001$ , partial  $\eta^2 = .710$ , 40mg/kg animals had lower BPM compared to 10mg/kg and controls, as well as 80mg/kg showing

considerably reduced BPM compared to control, 10mg/kg, and 20mg/kg groups. Animals administered 80mg/kg showed lower BPM, other than 40mg/kg at 2.5hr, compared to all other doses at 2hr,  $F(4, 25) = 11.792, p < .001$ , partial  $\eta^2 = .654$ , 2.5hr,  $F(4, 25) = 10.648, p < .001$ , partial  $\eta^2 = .630$ , and 3hr timepoints,  $F(4, 25) = 8.942, p < .001$ , partial  $\eta^2 = .589$ .

**Breath Rate.** Nearly all SADs have some impact on breath rate and can increase the probability of apneic episodes in neonates. These effects are often drug- and dose-dependent, with caffeine being the most commonly used preventative treatment. Interestingly, previous research reported baseline breath rate for PND6/7 C57BL/6 mice was approximately  $270 \pm 26$  BrPM (Zehendner et al., 2013). Although this may be a difference in mouse strain, pulse oximetry measures shown here are approximately 50% lower than these data. This may be explained by different techniques used to measure breath rate.

No significant Dose  $\times$  Time interaction was detected but a significant main effect of Time was found,  $F(3.828, 95.710) = 13.028, p < .001$ , partial  $\eta^2 = .343$ . There was a decline in BrPM from 30min to 3hr after injection when collapsed across dose,  $p < .01$  for all. Similarly, BrPM was higher at the 5min timepoint compared to 1hr, 1.5hr, and 2hr timepoints,  $p < .05$  for all. Despite these findings, Figure 3C illustrates a distinctive drop in BrPM for all groups administered BRX by 1hr post-injection. Further analysis revealed a significant between-subject main effect of Dose,  $F(4, 25) = 7.106, p = .001$ , partial  $\eta^2 = .532$ . Tukey's HSD post hoc analysis showed BrPM of animals in the control and 10mg/kg groups had higher BrPM compared to all other groups,  $p < .05$  for all.

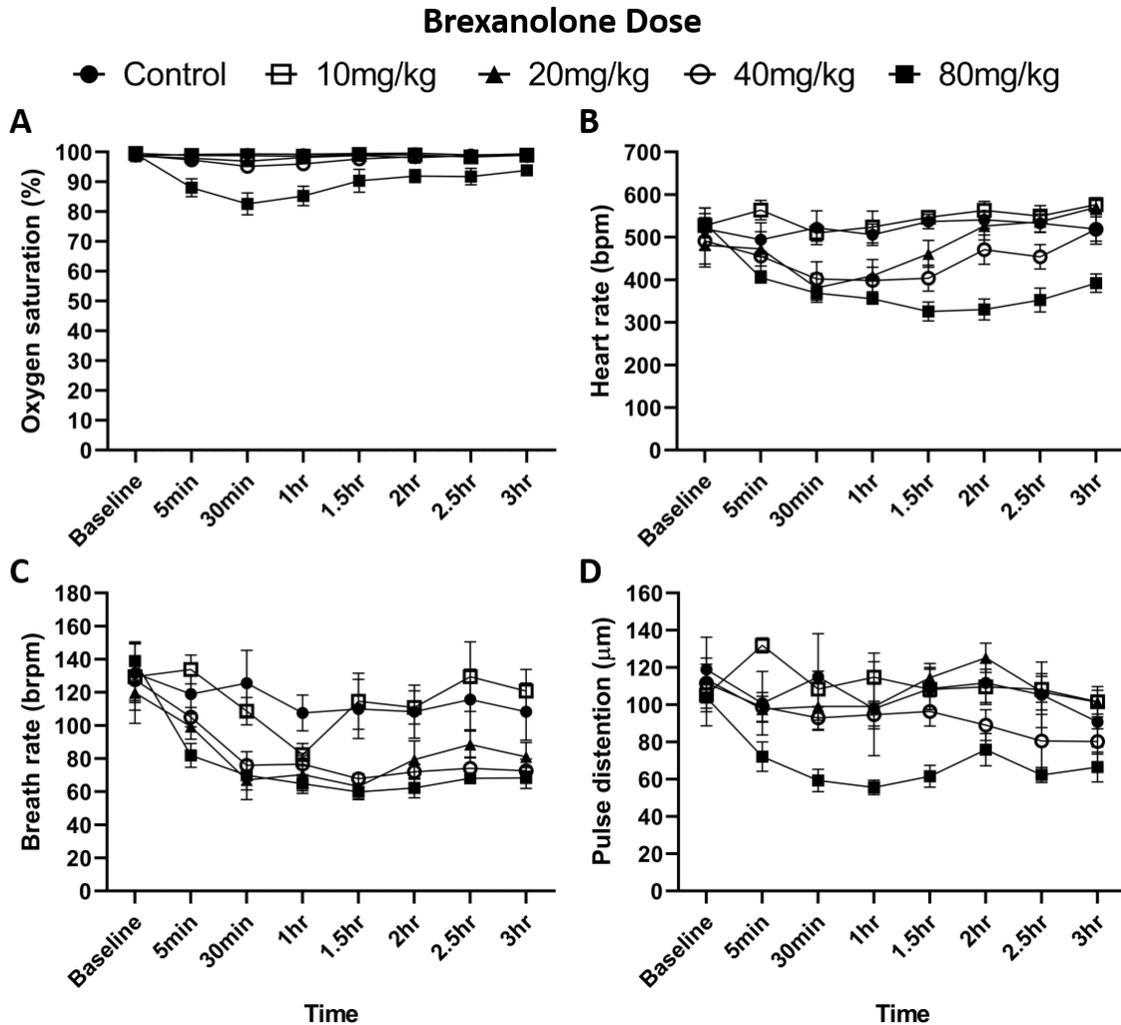


Figure 3. Dose-dependent effects of a single injection of captisol vehicle or BRX (10, 20, 40, or 80mg/kg) on vital signs measured by pulse oximetry once every 30min for 3hr. Primary vitals measures throughout the experiment included (A) peripheral oxygen saturation, (B) heart rate, (C) breath rate, and (D) pulse distention.

**Pulse Distention.** Pulse oximetry can be used to measure changes in the movement of arterial blood by fluctuations in light absorption. Drops in PD signal a reduction in blood flow at the point of measure, giving a rough estimation of arterial pressure / cardiac output. No significant Dose  $\times$  Time interaction was detected. Results showed a significant main effect of Time,  $F(7, 175) = 3.100$ ,  $p = .004$ , partial  $\eta^2 = .110$ , pointing to a statistically significant drop in PD at 3hr after injection compared to

baseline measures,  $p = .016$ . Between-subject analysis revealed a significant main effect of Dose,  $F(4, 25) = 4.996$ ,  $p = .004$ , partial  $\eta^2 = .444$ . Examination of Tukey's HSD post hoc values indicated PD for animals in the 80mg/kg group were significantly more impaired compared to control, 10 and 20mg/kg BRX groups,  $p < .05$  for all.

### ***Midazolam Reduces Heart and Breath Rate at Low-to-High Doses***

**Oxygen Saturation.** Early studies have shown MDZ produced transient fluctuations in blood oxygen levels (FDA 2017). However, doses used in humans are considerably lower than those able to produce sedation in neonatal rodents, likely due to the inability of IV administration and variable metabolic clearance of drug metabolites. Thus, all values are observed in neonatal mice administered drug IP. Results revealed no significant main effects. Statistical examination revealed minimal to no influence of MDZ dose. However, qualitative evaluation of the data suggests higher doses of MDZ at 60mg/kg and 120mg/kg can rapidly reduce SpO<sub>2</sub> to below 92% and 91%, respectively, risking induction of hypoxemia (Figure 4A).

**Heart Rate.** Fluctuations in heart rate can be easily produced when administering GABAergic drugs. Possible episodes of bradycardia, or abnormally low heart rate, pose risks for additional health complications and sudden death. There was a significant main effect of Time,  $F(7, 175) = 23.395$ ,  $p < .001$ , partial  $\eta^2 = .483$ , showing BPM decreased for all doses of MDZ. Additional inspection revealed a significant Dose  $\times$  Time interaction,  $F(28, 175) = 2.269$ ,  $p = .001$ , partial  $\eta^2 = .266$ , which was further analyzed by simple main effects.

Within-subject analysis of simple main effects demonstrated significant changes in BPM for every dose of MDZ. No differences were detected for saline injected controls,

$p = .964$ . Animals in the 15mg/kg group had higher BPM,  $F(7, 19) = 8.744$ ,  $p < .001$ , partial  $\eta^2 = .763$ , at baseline compared to all other timepoints excluding the final 3hr measurement,  $p < .05$  for all. Heart rate was also lower at 1hr, 1.5hr, and 2hr timepoints compared to 3hr post-injection, suggesting BPM was mostly restored by the end of experimental testing in the 15mg/kg group,  $p \leq .05$  for all. Significant within-subject differences were found for the 30mg/kg MDZ group,  $F(7, 19) = 8.060$ ,  $p < .001$ , partial  $\eta^2 = .748$ . There was an apparent decline in BPM by 30min, 1hr, and 2hr timepoints compared to baseline measures,  $p < .05$  for all. No other drops in BPM were detected, although the lowest reduction in BPM at 1hr and 2hr approached significance when compared to the 3hr timepoint,  $p = .054$  for all. Heart rate of 60mg/kg treated animals was higher,  $F(7, 19) = 5.830$ ,  $p = .001$ , partial  $\eta^2 = .682$ , at baseline compared to all timepoints other than the final 3hr measurement,  $p < .05$  for all. The spike in BPM recorded at 30min post-injection was also significantly different compared to the lowest measure of BPM for animals in the 60mg/kg group,  $p = .028$ . As expected, a significant reduction in BPM,  $F(7, 19) = 6.496$ ,  $p = .001$ , partial  $\eta^2 = .705$ , was revealed for animals administered 120mg/kg MDZ, in which all timepoints after injection were lower than baseline measures,  $p \leq .01$ .

There was a significant between-subject main effect of Dose,  $F(4, 25) = 9.745$ ,  $p < .001$ , partial  $\eta^2 = .609$ . Examination of simple main effects of the interaction revealed no significant difference between dose groups at baseline but did indicate a significant between-group difference 5min after injection,  $F(4, 25) = 6.549$ ,  $p = .001$ , partial  $\eta^2 = .512$ . Animals administered the highest doses of 60 and 120mg/kg MDZ showed a rapid and marked decrease in BPM when compared to controls,  $p = .002$  for all. By 30min

post-injection, animals treated with 30mg/kg MDZ exhibited lower BPM relative to controls,  $F(4, 25) = 3.626$ ,  $p = .018$ , partial  $\eta^2 = .367$ , although the effect of MDZ at this timepoint was similar for all doses,  $p = .041$ . All doses exhibited lower BPM by 1hr,  $F(4, 25) = 10.126$ ,  $p < .001$ , partial  $\eta^2 = .618$ , 1.5hr,  $F(4, 25) = 7.203$ ,  $p = .001$ , partial  $\eta^2 = .535$ , 2hr,  $F(4, 25) = 9.912$ ,  $p < .001$ , partial  $\eta^2 = .613$ , and 2.5hr,  $F(4, 25) = 5.793$ ,  $p = .002$ , partial  $\eta^2 = .481$ , after initial injection. One exception to these results includes the 30mg/kg group, illustrated by a spike in BPM at the 2hr timepoint that was maintained as statistically nonsignificant for the remaining measurements (Figure 4B). The final pulse oximetry measures yielded a significant difference in BPM,  $F(4, 25) = 11.413$ ,  $p < .001$ , partial  $\eta^2 = .646$ . Animals treated with 60mg/kg MDZ showed lower BPM relative to control, 15 and 30mg/kg groups. Similarly, those in the 120mg/kg group had lower BPM compared to control and 30mg/kg animals, although qualitative review concluded the outcome of 60 and 120mg/kg was very similar at the 3hr timepoint.

**Breath Rate.** Although MDZ is considered relatively safe to use in the NICU and pediatric medicine, some cases are reported in which respiratory depression from BZDs can induce apnea. There was a significant main effect of Time,  $F(3.836, 95.904) = 42.566$ ,  $p < .001$ , partial  $\eta^2 = .630$ , showed BrPM declined for all doses of MDZ (Figure 4C). Interestingly, this decline was also apparent for saline controls. A weak but significant Dose  $\times$  Time interaction,  $F(15.345, 95.904) = 1.773$ ,  $p = .049$ , partial  $\eta^2 = .221$ , was further examined for simple main effects.

Within-subject analysis of simple main effects revealed the BrPM of animals in the control group decreased throughout the experiment,  $F(7, 19) = 6.061$ ,  $p = .001$ , partial  $\eta^2 = .691$ . Breath rate at 5min post-injection was significantly different compared to 2hr,

2.5hr, and 3hr timepoints,  $p < .05$  for all. Additionally, the slight rise in BrPM at 1.5hr after injection was significantly higher than 3hr measurements,  $p = .012$ . Animals treated with 15mg/kg MDZ similarly showed a drop in BrPM,  $F(7, 19) = 7.245$ ,  $p < .001$ , partial  $\eta^2 = .727$ , relative to all timepoints after 5min post-injection,  $p < .05$  for all. The effect of 30mg/kg MDZ was slightly less robust,  $F(7, 19) = 4.030$ ,  $p = .007$ , partial  $\eta^2 = .598$ , but demonstrated impairment to BrPM by 2hr, 2.5hr, and 3hr timepoints relative to baseline measures,  $p < .05$  for all. A similar but more apparent outcome was also produced in animals administered 60mg/kg MDZ,  $F(7, 19) = 7.081$ ,  $p < .001$ , partial  $\eta^2 = .723$ . Breath rate was lower at every timepoint 1hr after injection when compared to baseline measures,  $p < .01$  for all. The reduction in BrPM 5min after injection was also lower when compared to the 2hr timepoint,  $p = .007$ . As one would expect, 120mg/kg MDZ produced the most robust impairment to BrPM,  $F(7, 19) = 6.396$ ,  $p = .001$ , partial  $\eta^2 = .702$ . From 5min to 3hr following injection, animals in the 120mg/kg group displayed markedly lower BrPM compared to baseline measures,  $p < .05$  for all.

There was a significant between-subject main effect of Dose,  $F(4, 25) = 4.628$ ,  $p = .006$ , partial  $\eta^2 = .425$ . Examination of simple main effects revealed no significant difference between dose groups at baseline. Significant between-group differences 5min after injection,  $F(4, 25) = 4.486$ ,  $p = .007$ , partial  $\eta^2 = .418$ , revealed animals administered 120mg/kg had lower BrPM compared to controls, suggesting rapid reductions in breath rate can be induced by high doses of MDZ,  $p = .004$ . By 1.5hr after injection,  $F(4, 25) = 6.356$ ,  $p = .001$ , partial  $\eta^2 = .506$ , all doses of MDZ produced significantly lower BrPM compared to controls,  $p < .05$  for all. Animals in the 120mg/kg MDZ group exhibited significantly lower BrPM compared to controls at 2hr,  $F(4, 25) =$

3.713,  $p = .017$ , partial  $\eta^2 = .373$ , 2.5hr,  $F(4, 25) = 4.068$ ,  $p = .011$ , partial  $\eta^2 = .394$ , and 3hr timepoints,  $F(4, 25) = 4.767$ ,  $p = .005$ , partial  $\eta^2 = .433$ . Importantly, the decrease in BrPM overtime for controls should be considered when interpreting these data. One explanation could be autonomic reactivity during initial stages of pulse oximetry. Decreased BrPM could be partly explained by habituation to the stress of testing.

All doses of MDZ produced a similar trend of weakened breath rate. Unlike most other pulse oximetry measures, animals at even the lowest dose of 15mg/kg appeared to not recover from the effects of MDZ, despite restoration to SpO<sub>2</sub>, BPM, and only slight changes to PD (see below).

**Pulse Distention.** As seen with most GABAergic drugs, cardiac output is typically reduced during sedation or anesthesia and may be detectable by changes in PD using pulse oximetry. There was a significant main effect of Time,  $F(7, 175) = 5.959$ ,  $p < .001$ , partial  $\eta^2 = .192$ , revealing a change in PD for 60 and 120mg/kg MDZ. A weak but significant Dose  $\times$  Time interaction was found,  $F(28, 175) = 1.567$ ,  $p = .044$ , partial  $\eta^2 = .200$ .

Within-subject analysis for simple main effects showed no significant difference in control, 15 and 30mg/kg groups. Interestingly, animals administered 60mg/kg exhibited a significant increase in PD,  $F(7, 19) = 4.024$ ,  $p = .007$ , partial  $\eta^2 = .597$ . However, upon further examination of simple main effects, no statistically significant differences were detected. Animals in the 60mg/kg group approached a significant difference between baseline and 5min post-injection,  $p = .076$ . There was a statistically significant difference in PD for animals treated with 120mg/kg MDZ,  $F(7, 19) = 5.997$ ,  $p = .001$ , partial  $\eta^2 = .688$ . A rapid increase in PD occurred from baseline to 5min

following injection,  $p < .001$ . This spike in PD was also significantly greater relative to PD values between 1hr–2.5hr after injection,  $p < .05$  for all. Between-subject analysis yielded no differences between dose groups. Despite these findings, qualitative examination of Figure 4D suggests MDZ increases PD relative to controls, regardless of dose.

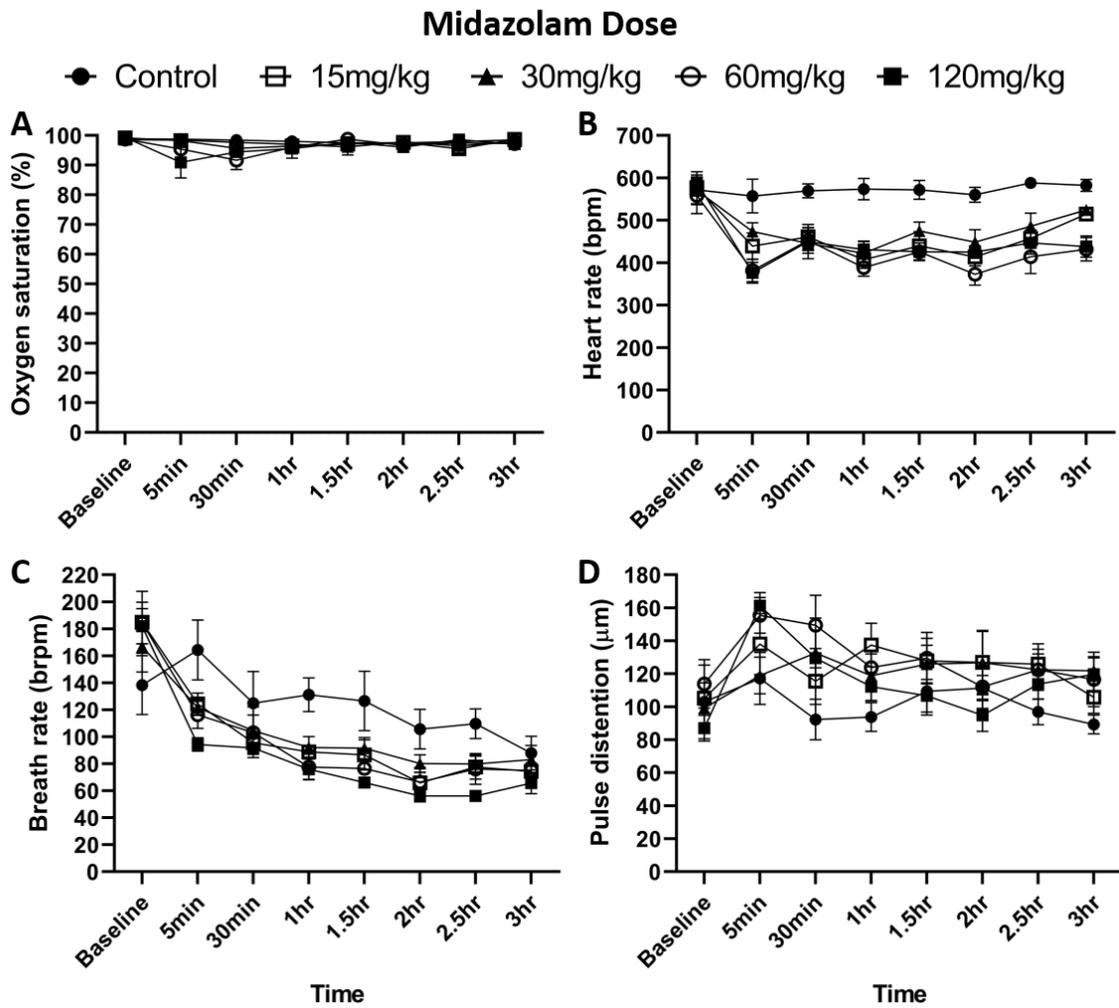


Figure 4. Dose-dependent effects of a single injection of MDZ on vital signs measured by pulse oximetry once every 30min for 3hr. Primary vitals measures throughout the experiment included (A) peripheral oxygen saturation, (B) heart rate, (C) breath rate, and (D) pulse distention.

***Brexanolone Induces Minimal Neurotoxicity at Low Doses***

Brexanolone was administered as a single IP injection across of a range of low-to-high doses to examine the neuroapoptotic effect of AlloP in the developing rodent brain. There was a significant main effect of dose on mean density of apoptotic cells per  $\text{mm}^3$ ,  $F(4, 15) = 10.726$ ,  $p < .001$ , partial  $\eta^2 = .741$ . Tukey's HSD post hoc analysis revealed animals administered 40mg/kg BRX had greater apoptotic profiles compared to controls,  $p = .001$ , 10mg/kg,  $p = .009$ , and 20mg/kg,  $p = .029$ , groups. The highest dose of 80mg/kg also yielded more neurotoxicity compared to controls,  $p = .001$ , 10mg/kg,  $p = .015$ , and 20mg/kg,  $p = .047$ , groups.

Importantly, no statistically significant changes in neurotoxicity were detected between animals administered captisol vehicle, 10 and 20mg/kg BRX, suggesting the use of BRX to induce acute sedation can be delivered without the risk of neuroapoptosis. The apoptogenicity of BRX peaked by 40mg/kg, with slightly more apoptotic profiles compared to the 80mg/kg group.

***Midazolam is Neurotoxic at Moderate-to-High Doses***

The pro-apoptotic impact of MDZ at low doses has been previously shown (Young et al., 2005), but how this effect scales with very high doses has yet to be examined. There was a significant effect of dose on mean density of apoptotic cells per  $\text{mm}^3$ ,  $F(4, 15) = 14.300$ ,  $p < .001$ , partial  $\eta^2 = .792$ . Tukey's HSD post hoc analysis animals administered 30mg/kg MDZ had more apoptotic profiles compared to saline controls,  $p = .002$ . MDZ at 60mg/kg displayed greater neurotoxicity compared to saline controls,  $p < .001$ , and 15mg/kg,  $p = .013$ , groups. Additionally, animals administered 120mg/kg MDZ showed significantly greater AC3-positives profiles compared to saline

controls,  $p < .001$ , and 15mg/kg,  $p = .007$ , groups. The apoptogenicity of MDZ appeared to peak by 60mg/kg, showing no apparent differences in apoptotic profiles despite twice the dose.

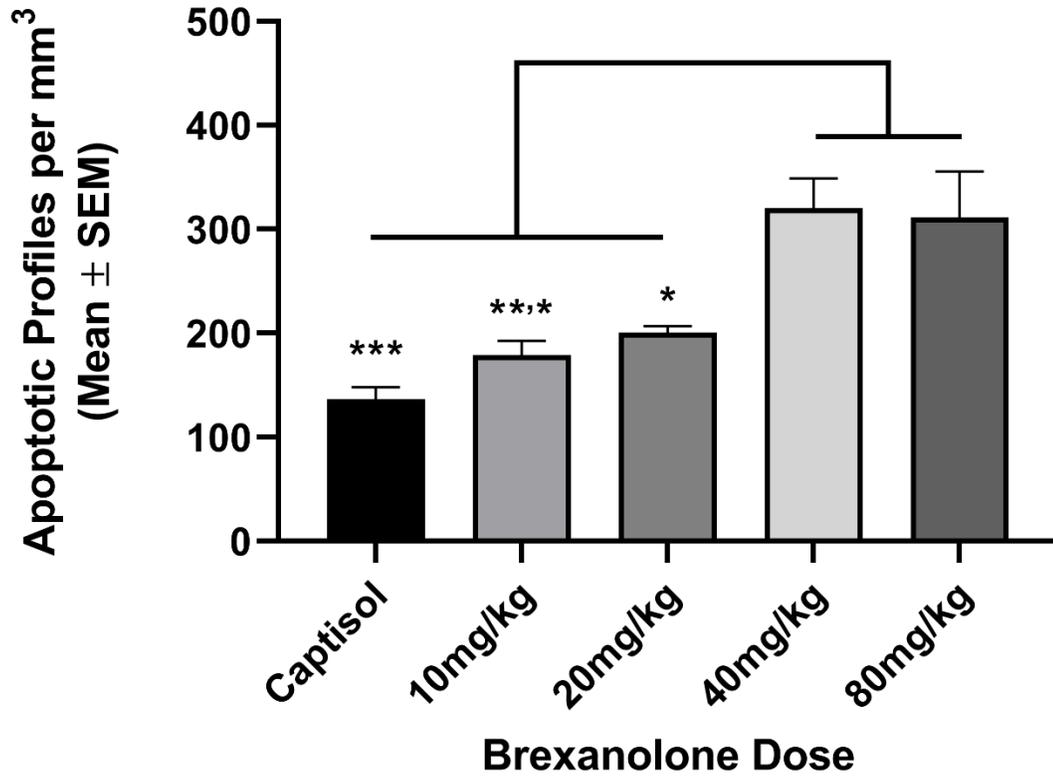


Figure 5. Mean apoptotic profiles per mm<sup>3</sup> for captisol vehicle and BRX doses in PND7 mice 6hr after a single IP injection. No significant differences in AC3-positive profiles were detected between captisol vehicle, 10 and 20mg/kg BRX groups, indicating sedative doses of BRX are non-neurotoxic. Doses at 40 and 80mg/kg yielded significantly more apoptosis compared to animals administered captisol vehicle, 10 and 20mg/kg BRX. Doses at 40 and 80mg/kg also produced near AC3-positive profiles, suggesting a threshold for BRX's apoptogenicity in the developing rodent brain lies between 20 and 40mg/kg. \*\*\* =  $p \leq .001$ , captisol compared to 40 and 80mg/kg; \*\* =  $p < .01$ , 10mg/kg compared to 40mg/kg; \* =  $p < .05$ , 10mg/kg compared to 80mg/kg and 20mg/kg compared to 40 and 80mg/kg.

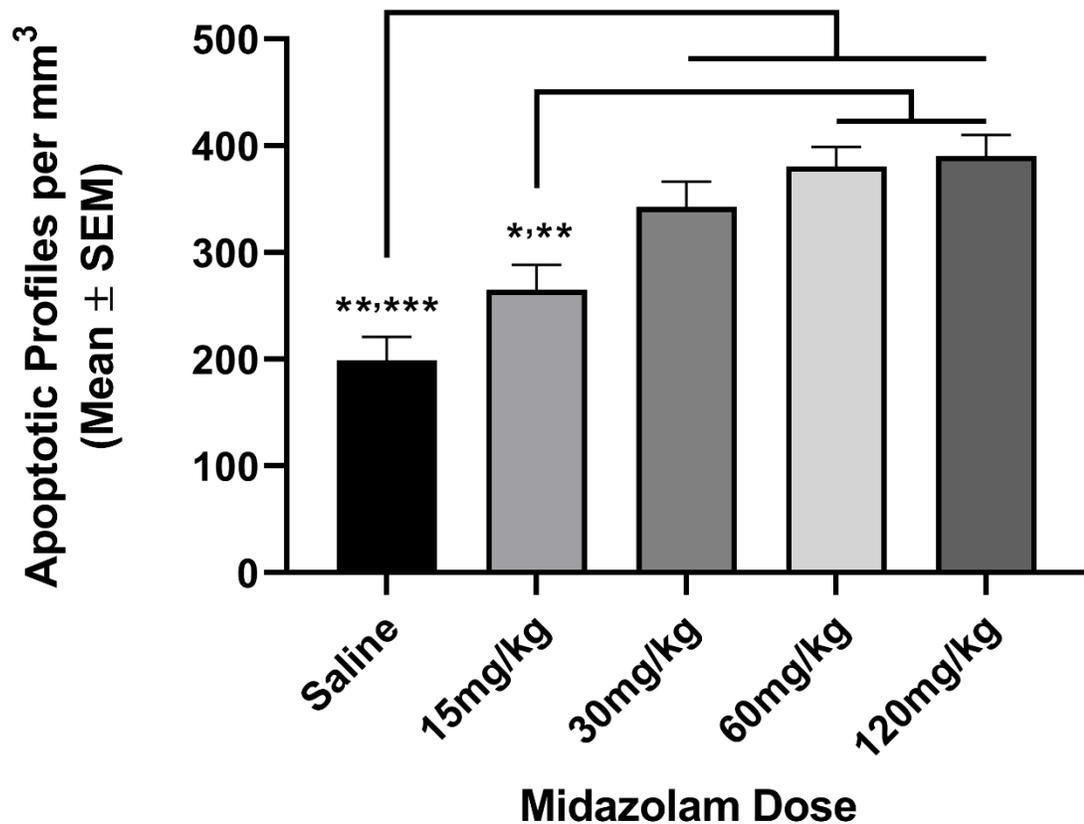


Figure 6. Mean apoptotic profiles per mm<sup>3</sup> for saline vehicle and MDZ doses in PND7 mice 6hr after a single IP injection. No significant differences in AC3-positives profiles were detected between saline controls and 15mg/kg, as well as 15 and 30mg/kg MDZ. Animals administered 30, 60, and 120mg/kg MDZ had significantly more apoptotic profiles compared to saline controls, indicating even mild sedation by MDZ is neurotoxic. No significant differences in AC3-positives profiles was detected between 30, 60, and 120mg/kg treated animals, suggesting the peak neurotoxicity of a single exposure to MDZ can be obtained with relatively low doses. \*\*\* =  $p < .001$ , saline compared to 60 and 120mg/kg; \*\* =  $p < .01$ , saline compared to 30mg/kg and 15mg/kg compared to 120mg/kg; \* =  $p < .05$ , 15mg/kg compared to 60mg/kg.

#### *Dose-response Curves for Neurotoxicity of Brexanolone and Midazolam*

Maximum neurotoxic effect of both drugs was obtained with the two highest doses, revealing a maximum potential apoptogenicity of BRX and MDZ. The ED<sub>50</sub> for estimated population of apoptotic cells produced by BRX was 23.3mg/kg (95% CI, 16.81 – 30.26;  $R$ -square = .8869) while the ED<sub>50</sub> for MDZ was 15.7mg/kg (95% CI, 14.21-17.44;  $R$ -square = .9711), indicating 50% of the maximum neurotoxic capability of BRX

requires a higher dose relative to MDZ. Additionally, MDZ-induced neurotoxicity was qualitatively greater ( $Top = 52,357$  apoptotic cells) compared to BRX ( $Top = 45,285$  apoptotic cells) by evaluation of overall upper limits.

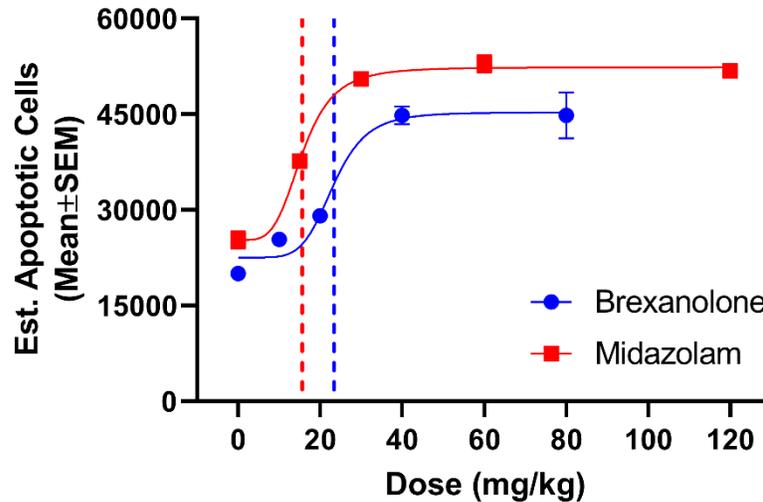


Figure 7. Dose-response curves for neurotoxic effect of BRX and MDZ in PND7 mice 6hr after single IP injection. Dashed lines indicate ED<sub>50</sub> for neurotoxic effect of each drug measured by total estimated apoptotic cells. The ED<sub>50</sub> for neurotoxicity was 23.3mg/kg for BRX and 15.7mg/kg for MDZ. The neurotoxic effect of both drugs plateaued by the final two doses, indicating a maximal apoptotic effect in the developing brain when processed 6hr after exposure.

## Experiment 2

### *Brexanolone Delivers Similar Prolonged Sedation Compared to Midazolam*

Evaluation of the behavioral indices determining the level of sedation is not possible during the continuous infusion procedure since each animal is secured to a platform. Motion detection was used as a detailed, high frequency method of monitoring general motor activity of the animals. The presence of motion indicates these were sedative and not anesthetic doses, as no movement would be detectable during total anesthesia. There was a significant main effect of Time,  $F(2.423, 24.235) = 18.930, p < .001$ , partial  $\eta^2 = .654$ . Further examination revealed a significant Drug  $\times$  Time

interaction,  $F(2.423, 24.235) = 4.211$ ,  $p = .021$ , partial  $\eta^2 = .296$ , which was analyzed by simple main effects.

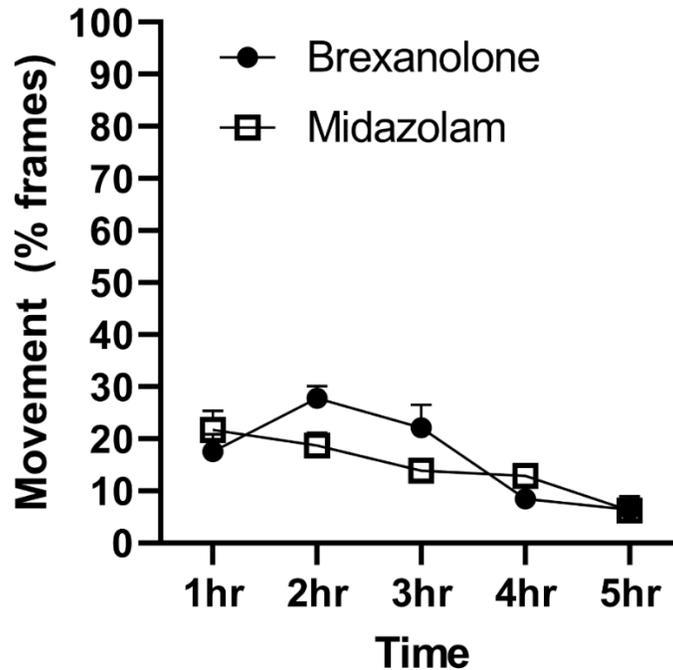


Figure 8. Plotted motion detected for BRX (20mg/kg bolus + 10mg/kg/hr) and MDZ (30mg/kg bolus + 15mg/kg/hr) every hour until equal sedation, approx. 4 to 5 hrs. No significant differences were detected between BRX and MDZ treatments, indicating relatively similar levels of sedation were obtained by the infusion procedure.

Within-subject analysis of simple main effects showed a decrease in detected motion over the experimental timepoints for animals infused with BRX,  $F(4, 7) = 10.089$ ,  $p = .005$ , partial  $\eta^2 = .852$ , and MDZ,  $F(4, 7) = 8.703$ ,  $p = .008$ , partial  $\eta^2 = .833$ . Animals in the BRX group showed less movement by the 4<sup>th</sup> and 5<sup>th</sup> hours of infusion compared to the 2<sup>nd</sup> and 3<sup>rd</sup> hour measurements,  $p < .05$  for all, but no statistically significant difference compared to the 1<sup>st</sup> hour. Thus, the BRX group may have become less sedated during the 2<sup>nd</sup> and 3<sup>rd</sup> hours of infusion as effects from the bolus wore off, followed by steadied sedation during the 4<sup>th</sup> and 5<sup>th</sup> hours. Animals in the MDZ group were more

sedated by the 5<sup>th</sup> hour compared to the 1<sup>st</sup>, 2<sup>nd</sup>, and 4<sup>th</sup> hours,  $p < .05$  for all. Despite slight fluctuations within-treatment during infusions, no significant between-subject effects were found for the Drug condition,  $F(1, 10) = 0.429$ ,  $p = .527$ , partial  $\eta^2 = .041$ . Importantly, this indicated reasonably similar levels of sedation were obtained from both drug treatments over the course of infusion (Figure 8). The overall reduction in motion for both groups might be partly explained by habituation to the infusion procedure.

### ***Midazolam Weakens Heart and Breath Rate More Than Brexanolone***

**Oxygen Saturation.** Continuous infusion of MDZ at low dosages is employed in the NICU and helps to maintain light sedation over prolonged periods to prevent agitation of neonatal patients. Results for SpO<sub>2</sub> revealed there was no significant within-subject main effect of Time,  $F(4.666, 60.663) = 1.278$ ,  $p = .287$ , partial  $\eta^2 = .089$ , between-subject main effect of Drug,  $F(2, 13) = 0.236$ ,  $p = .793$ , partial  $\eta^2 = .035$ , or Time  $\times$  Drug interaction,  $F(9.333, 60.663) = 1.180$ ,  $p = .323$ , partial  $\eta^2 = .154$ . Illustrated in Figure 9A, these findings indicate the effects of prolonged exposure to an equally sedative regimen of MDZ and BRX do not negatively impact SpO<sub>2</sub>.

**Heart Rate.** There was a significant main effect of Time,  $F(4.048, 52.623) = 3.282$ ,  $p = .017$ , partial  $\eta^2 = .202$ , as well as a significant Time  $\times$  Drug interaction was found,  $F(8.096, 52.623) = 4.602$ ,  $p < .001$ , partial  $\eta^2 = .415$ , suggesting changes in BPM over the course of sedation were influenced by the drug administered. Within-subject examination of simple main effects indicated a decrease in BPM for MDZ treated animals when comparing baseline to 30min and 3.5hr after the start of infusion,  $p < .05$  for all. This appears qualitatively negligible given the relatively high BPM at baseline for the MDZ group (Figure 9B).

Despite some detectable differences within-group, a between-subject main effect of Dose was found,  $F(2, 13) = 13.541$ ,  $p = .001$ , partial  $\eta^2 = .676$ , indicating animals in the BRX and MDZ group had lower BPM compared to controls,  $p = .031$  and  $p < .001$ , respectively. Examination of simple main effects showed a significant drop in BPM by 30min following start of infusion,  $F(2, 13) = 7.333$ ,  $p = .007$ , partial  $\eta^2 = .530$ , as well as from 1.5hr post-infusion until the end of testing,  $F(2, 13) = 5.850-40.023$ ,  $p = .007$  to  $p < .001$ , partial  $\eta^2 = .474-.860$ . More specifically, the initial dip in BPM for MDZ 30min after beginning infusion was lower than controls,  $p = .006$ . Animals in the MDZ group also had lower BPM compared to controls from 1.5hr to 6hr of infusion,  $p < .01$  for all. No significant difference between BRX and MDZ were detected until the final measurement at 6hr,  $p = .042$ . A statistically significant drop in BPM between BRX and controls was found starting at 2.5hr of infusion and continued from 3.5–5hr timepoints. Finally, BPM was lower between BRX and controls at the final 6hr measurement. No differences were detected between baseline measures.

Qualitative examination of Figure 9B suggests the drug effect on BPM were stabilized by approximately 1.5hr after the start of infusion. As such, the average BPM was compiled across 1.5–6hr of infusion for MDZ ( $M = 407.13$  BPM) and BRX ( $M = 489.45$  BPM) and were used to calculate the percentage of effect against controls ( $M = 592.84$  BPM). BRX animals showed an average of 17% lower BPM compared to controls, while the MDZ group had approximately 31% lower BPM. While the comparison between BRX and MDZ showed no between-subject differences other than measurements at hour 6, these data indicate a potential clinically significant effect that should be considered when interpreting these data.

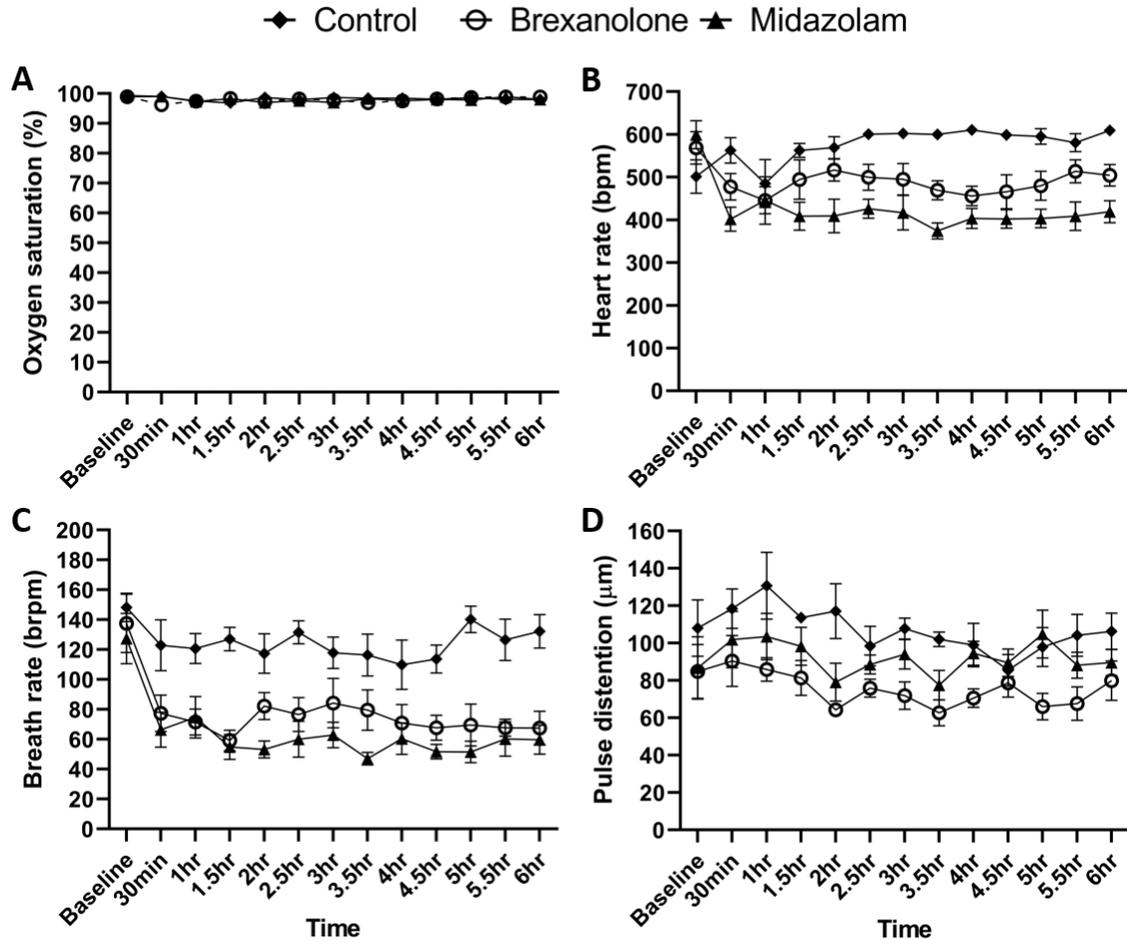


Figure 9. Effect of saline (single injection, vol. matched to bolus at 6µl/g), BRX (20mg/kg bolus + 10mg/kg/hr) and MDZ (30mg/kg bolus + 15mg/kg/hr) on vital signs in PND7 mice recorded by pulse oximetry during 6hr continuous IP infusion. Measurements were taken every 30min for 6 hours. Primary vitals measured throughout the experiment included (A) peripheral oxygen saturation, (B) heart rate, (C) breath rate, and (D) pulse distention.

**Breath Rate.** Result for monitored BrPM revealed no Time × Drug interaction,  $F(10.073, 65.473) = 1.188, p = .315, \text{partial } \eta^2 = .155$ . There was a significant main effect of Time,  $F(5.036, 65.473) = 7.366, p < .001, \text{partial } \eta^2 = .362$ , indicating baseline BrPM was higher compared to all other timepoints when collapsed across treatment groups,  $p < .05$  for all. Examination of between-subject effects revealed a significant main effect of Drug,  $F(2, 13) = 22.582, p < .001, \text{partial } \eta^2 = .776$ . Tukey's HSD post hoc analysis

indicated animals in the BRX and MDZ groups had significantly less BrPM compared to controls,  $p \leq .001$ . Although no statistically significant differences were detected between BRX and MDZ groups, BrPM of the BRX group appears qualitatively less compromised when examine alongside the MDZ group.

**Pulse Distention.** There was no significant main effect for Time or Time  $\times$  Drug interaction detected. A significant between-subject main effect was uncovered for Drug,  $F(2, 13) = 10.763$ ,  $p = .002$ , partial  $\eta^2 = .623$ . Tukey's HSD post hoc analysis revealed animals in the control group had significantly higher PD compared to the BRX group when collapsed across time. No differences were detected for MDZ compared to either treatment group. Qualitative examination of the dataset indicated animals in the BRX group had relatively lower PD compared to MDZ, suggesting BRX might have the unique side-effect of lowering cardiac output.

#### ***Brexanolone and Midazolam are Similarly Neurotoxic Following 6-hour Infusion***

Results from experiment 1 detail the minimal neurotoxic effects of sufficiently sedative, acute doses of BRX but high pro-apoptotic impact following mild sedation with MDZ. As such, I examined whether similarly low apoptogenicity was obtainable following continuous infusions of comparably sedative doses of BRX and MDZ (Figure 10).

There was a significant effect of drug treatment on mean density of apoptotic cells per  $\text{mm}^3$ ,  $F(3, 23) = 13.400$ ,  $p < .001$ , partial  $\eta^2 = .636$ . Tukey's HSD post hoc analysis revealed animals in the BRX condition had higher apoptotic profiles compared to saline and captisol controls,  $p = .003$  and  $p = .002$ , respectively. Similarly, MDZ treated animals had higher density of AC3+ cells compared to saline and captisol controls,  $p < .001$  for

all. This suggests that, despite generating less neuroapoptosis following acute injection of low doses, BRX resulted in relatively similar levels of neurotoxicity compared to MDZ following continuous infusion.

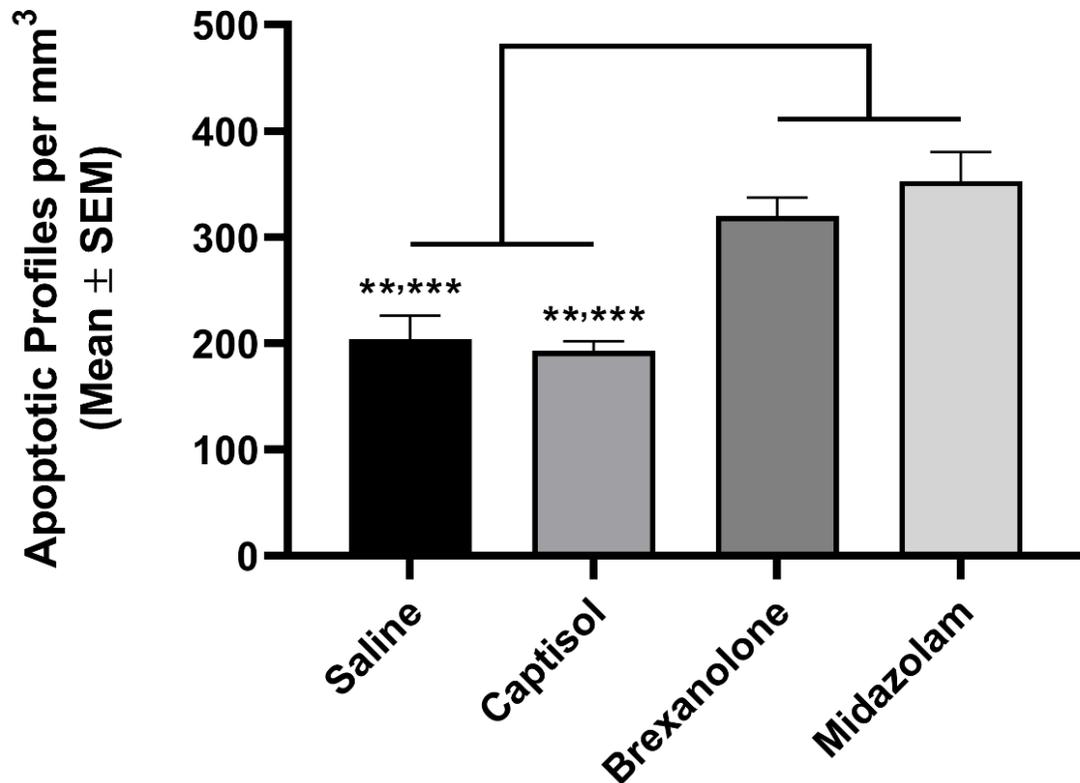


Figure 10. Mean apoptotic profiles per mm<sup>3</sup> for saline (single injection vol. matched to bolus at 6µl/g), captisol (single injection vol. matched to bolus at 4µl/g), BRX (20mg/kg bolus + 10mg/kg/hr), and MDZ (30mg/kg bolus + 15mg/kg/hr) in PND7 mice following 6hr of continuous IP infusions. No significant differences in AC3-positive profiles were detected between saline and captisol controls, as well as animals infused with BRX compared to MDZ. However, continuous infusion with BRX and MDZ yielded significantly greater apoptosis profiles compared to saline and captisol controls, suggesting both drugs had similar apoptogenicity after prolonged exposure. \*\*\* =  $p < .001$ , saline and captisol compared to MDZ; \*\* =  $p < .01$ , saline and captisol compared to BRX.

## Supplemental Data Analyses

### *Acute Brexanolone is Less Neurotoxic Than Equally Sedative Doses of Midazolam*

Dose-response data in Figures 2 and 7 indicated MDZ would not generate clinically relevant levels of sedation without a significant neurotoxic effect. Based on pulse oximetry data presented in Figure 4, the extent of MDZ's impact to most vital signs occurred between 5min and 30min after injection. The same effect was observed with BRX from 30min to 1hr after injection, suggesting MDZ may be faster acting than BRX. Thus, the sedative window of MDZ could have been overlooked by beginning behavioral observations 30min after injection. Movement was quantified for the first 30min of activity after injection of BRX or MDZ to examine whether a deeper sedative state went unnoticed.

Results revealed a significant difference in detected motion between BRX doses,  $F(3, 12) = 6.883$ ,  $p = .006$ , partial  $\eta^2 = .632$ . Figure 11A shows animals administered 40 and 80mg/kg moved less compared to the 10mg/kg group,  $p = .014$  and  $p = .007$ , respectively. No statistically significant differences in movement were detected between doses of MDZ. Paradoxically, 30mg/kg MDZ induced the greatest reduction in movement (Figure 11B).

The average percent movement was then compared across doses for BRX and MDZ to determine if relatively equal sedation was produced. BRX at 10mg/kg ( $M = 8.61\%$ ) displayed near identical reductions in movement compared to MDZ at 60mg/kg ( $M = 8.54\%$ ),  $t(9) = .014$ ,  $p = .989$ . Reductions in movement by 20mg/kg BRX ( $M = 4.61\%$ ) were also similar to MDZ at 120mg/kg ( $M = 5.83\%$ ),  $t(8) = -.346$ ,  $p = .738$ . These

data indicate BRX at 10 and 20mg/kg produced relatively equal sedation compared to 60 and 120mg/kg MDZ, respectively, during the first 30min of exposure (Figure 11C).

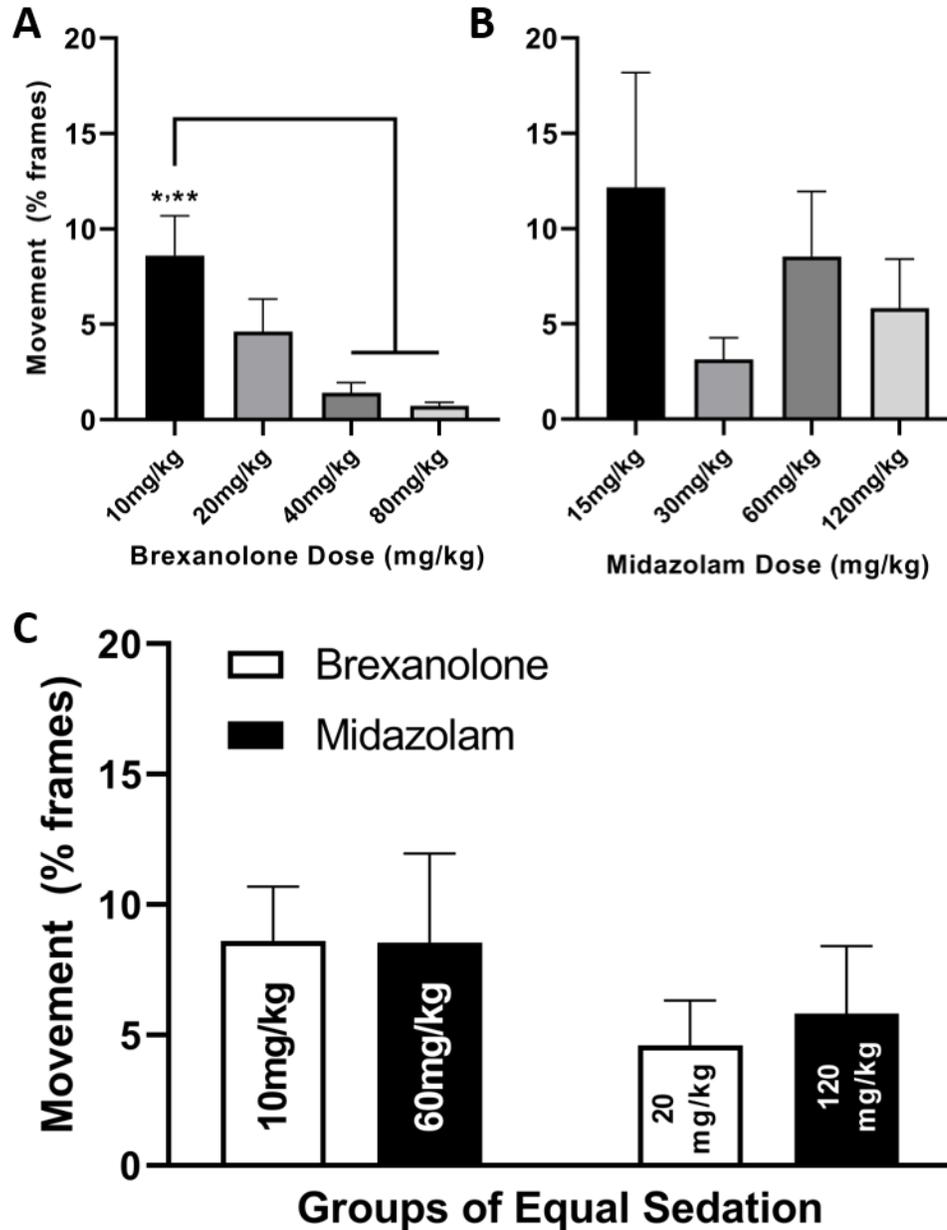


Figure 11. Plotted motion detected during the first 30min after a single injection of low to high doses of BRX or MDZ. (A) Dose dependent effect of BRX on movement. Doses of 40 and 80mg/kg significantly reduced detected motion compared to the 10mg/kg group. (B) No significant differences in movement were detected across MDZ doses. However, animals administered 30mg/kg displayed the greatest reduction in detected motion. (C) BRX at 10mg/kg produced equal sedation compared to 60mg/kg MDZ. Similarly, 20mm/kg BRX and 120mg/kg MDZ induced the same reduction in movement, indicating

a relatively equal sedative drug effect. \*\* =  $p < .01$ , (A) 10mg/kg compared to 80mg/kg BRX; \* =  $p < .05$ , (A) 10mg/kg compared to 40mg/kg BRX.

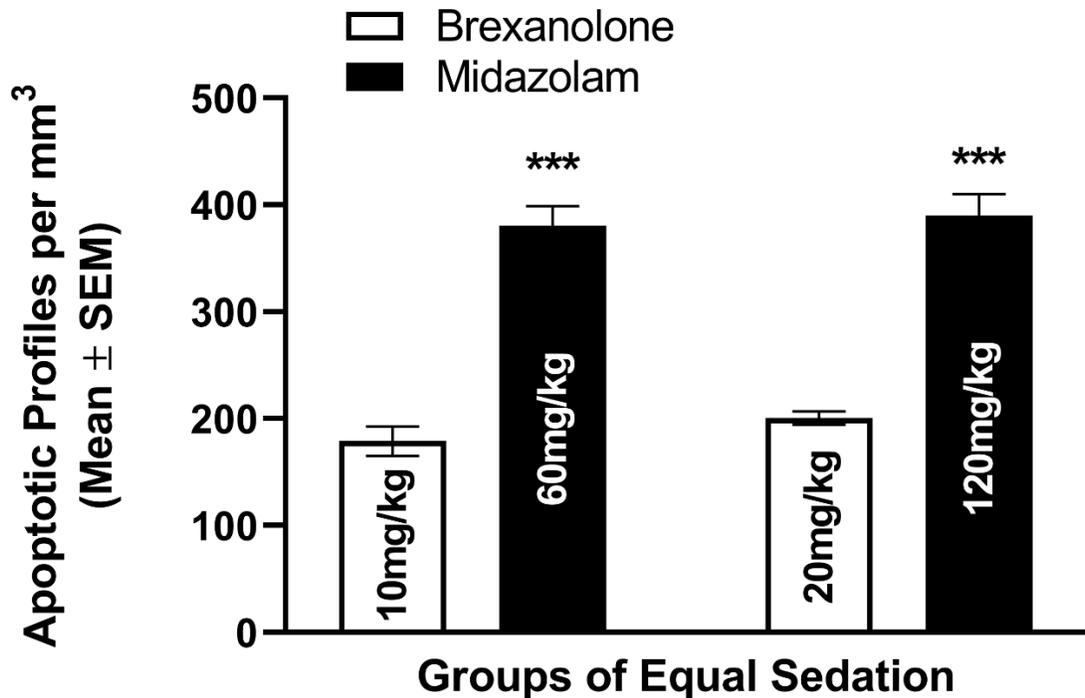


Figure 12. Mean apoptotic profiles per mm<sup>3</sup> of equally sedative doses of BRX and MDZ in PND7 mice. Results revealed 60mg/kg MDZ induced significantly more neuroapoptosis compared to 10mg/kg BRX. Similarly, MDZ at 120mg/kg produced a significantly greater neurotoxic effect compared to 20mg/kg BRX. These data suggest BRX produces considerably less neurotoxic compared to equally sedative doses of MDZ. \*\*\* =  $p < .001$ , BRX doses compared to MDZ doses.

Results from experiment 1 suggest low doses of BRX generate ample sedation with minimal neurotoxic consequences. To examine whether this stands in the context of equally sedative doses of MDZ, apoptotic profiles from experiment 1 were used to compare neurotoxicity (Figure 12). Animals administered BRX at 10mg/kg ( $M = 178.87$ ) showed significantly less mean density apoptotic profiles per mm<sup>3</sup> compared to an equally sedative dose of MDZ at 60mg/kg ( $M = 380.74$ ),  $t(6) = -8.816$ ,  $p < .001$ . Similarly, BRX at 20mg/kg ( $M = 200.50$ ) triggered far less neurotoxicity compared to an equally sedative dose of 120mg/kg MDZ ( $M = 390.12$ ),  $t(6) = -8.996$ ,  $p < .001$ . Taken

together, these results support the conclusion that BRX can deliver transient sedation with far less neurotoxic consequences than MDZ.

### ***Captisol May Reduce Physiological Neuroapoptosis***

Cyclodextrins may be protective against hypoxia-ischemia in young rats (Rivers et al., 2012). They are also neuroprotective in neonatal models of Niemann-Pick Type C (NP-C) Disease, a fatal neurodegenerative disease involving diminished cholesterol trafficking that prevents proper neurosteroidogenesis (Ottinger et al., 2014). Based on these data, I created a composite dataset of vehicle control groups from experiment 1 and 2 to determine whether captisol altered physiological neuroapoptosis. Drug administration and processing for control animals was the same for all groups, i.e. sacrifice 6hr after single injection. Control animals in experiment 1 received 7.5 $\mu$ l/g of either saline or captisol cyclodextrin, while those in experiment 2 received saline as 6 $\mu$ l/g or captisol at 4 $\mu$ l/g. Groups administered saline were computed as one treatment because: 1) there was a small difference in total volume, 1.5 $\mu$ l/g, 2) saline has not been shown to alter physiological neuroapoptosis, and 3) initial assessment of saline groups from experiment 1 ( $M = 198.90$ ) and 2 ( $M = 203.96$ ) revealed near equal mean density of apoptotic cells per  $\text{mm}^3$ .

Results from the composite dataset yielded a significant difference in mean density of apoptotic cells per  $\text{mm}^3$ ,  $F(2, 16) = 4.166$ ,  $p = .035$ , partial  $\eta^2 = .342$ . Tukey's HSD post hoc analysis revealed animals that received captisol at 7.5 $\mu$ l/g had significantly less physiological neuroapoptosis compared to saline controls,  $p = .030$ . No differences were detected between captisol doses,  $p = .107$ , or saline and 4 $\mu$ l/g captisol. These

findings are puzzling and indicate BRX's low apoptogenicity at sedative, non-neurotoxic doses may be partly attributed to captisol cyclodextrin.

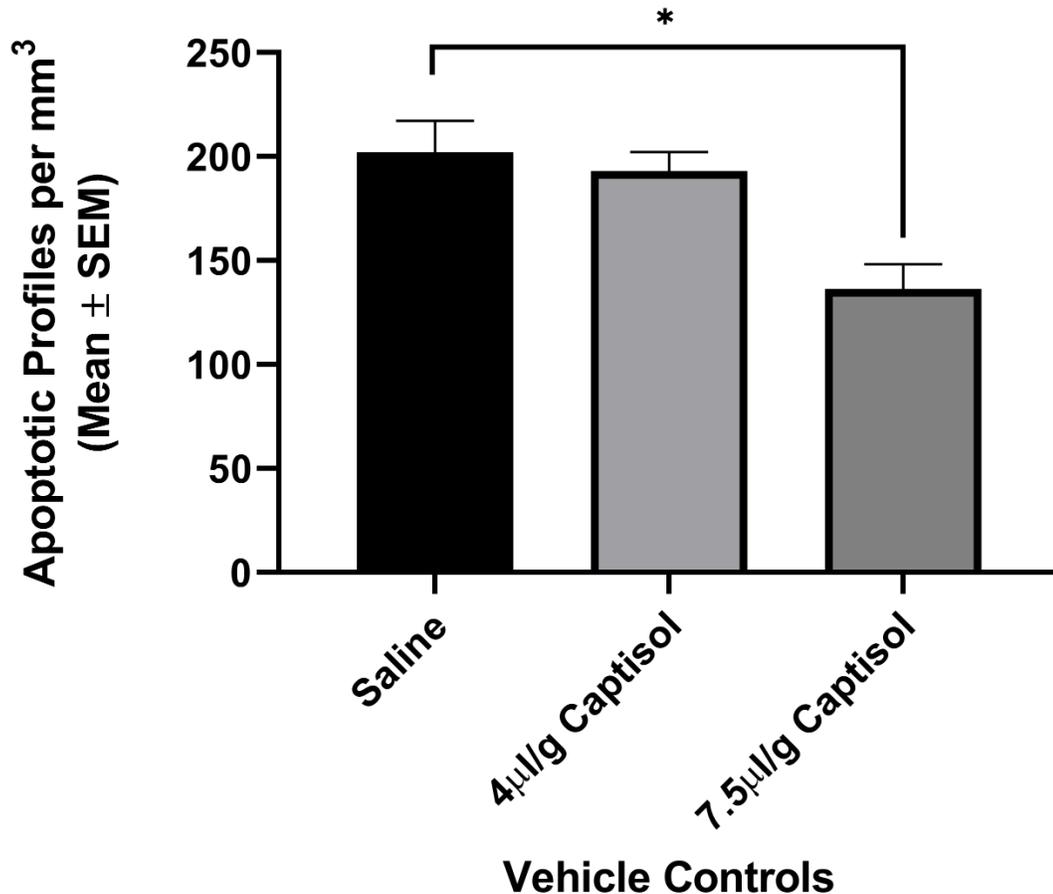


Figure 13. Mean density apoptotic profiles per mm<sup>3</sup> of a composite dataset for vehicle treated controls groups. Animals in the saline condition are from experiment 1 and 2 and received either 6 or 7.5µl/g 0.9% saline. Animals that received 7.5µl/g captisol were from experiment 1 while those administered 4µl/g captisol were part of experiment 2. Results yielded a significant reduction in neuroapoptosis in the 7.5µl/g captisol group compared to those treated with saline. No significant differences in AC3-positive profiles were detected between saline and 4µl/g captisol, or 4 and 7.5µl/g captisol groups. \* =  $p < .05$ , saline compared to 7.5µl/g captisol.

***Brexanolone and Midazolam Produce Analogous Neurotoxicity at the Same High-dose Given as a Single Injection or Continuous Infusion***

Although low doses of BRX were able to induce sedation with minimal neurotoxicity, high doses still produced a considerable increase in apoptotic profiles. There was also a similar neurotoxic effect between BRX and MDZ following 6hr continuous infusion. One explanation may be a threshold of neurotoxicity in which the actions of BRX eventually become apoptogenic. Importantly, dose-response data presented in Figure 7 suggests the neurotoxic effects of both BRX and MDZ effectively plateaued by 40 and 60mg/kg, respectively. Additionally, the highest doses of BRX and MDZ were the same quantity of drug administered during continuous infusion (bolus + drug per hour\*6). Neurotoxic profiles from 80mg/kg BRX were compared with those from continuous infusion to determine whether the neurotoxic effect of BRX was a consequence of slow and prolonged exposure or total quantity of drug administered. Included in the analysis were data from experiment 1 and 2 for 120mg/kg MDZ administered acutely and during continuous infusion. This was done to determine whether the neurotoxic plateau of BRX was different from MDZ.

Results revealed no significant difference in mean density of apoptotic cells per  $\text{mm}^3$  between all groups,  $F(3, 20) = 1.465$ ,  $p = .254$ , partial  $\eta^2 = .180$ . As shown in Figure 14, animals treated with MDZ showed marginally higher apoptotic profiles compared to BRX, but no statistically significant differences were detected. Interestingly, apoptotic profiles for animals treated with 80mg/kg BRX as a single injection ( $M = 311.34$ ) and continuous infusion ( $M = 319.84$ ) were nearly the same. As expected, independent t-test comparisons between single injection and continuous infusion per drug group yielded no

significant differences for MDZ or BRX,  $p = .376$  and  $p = .829$ , respectively. Taken together, these data demonstrate both drugs induce a similar plateau of neurotoxicity and that overall dose may be more important than duration of exposure when predicting neurotoxic outcomes.

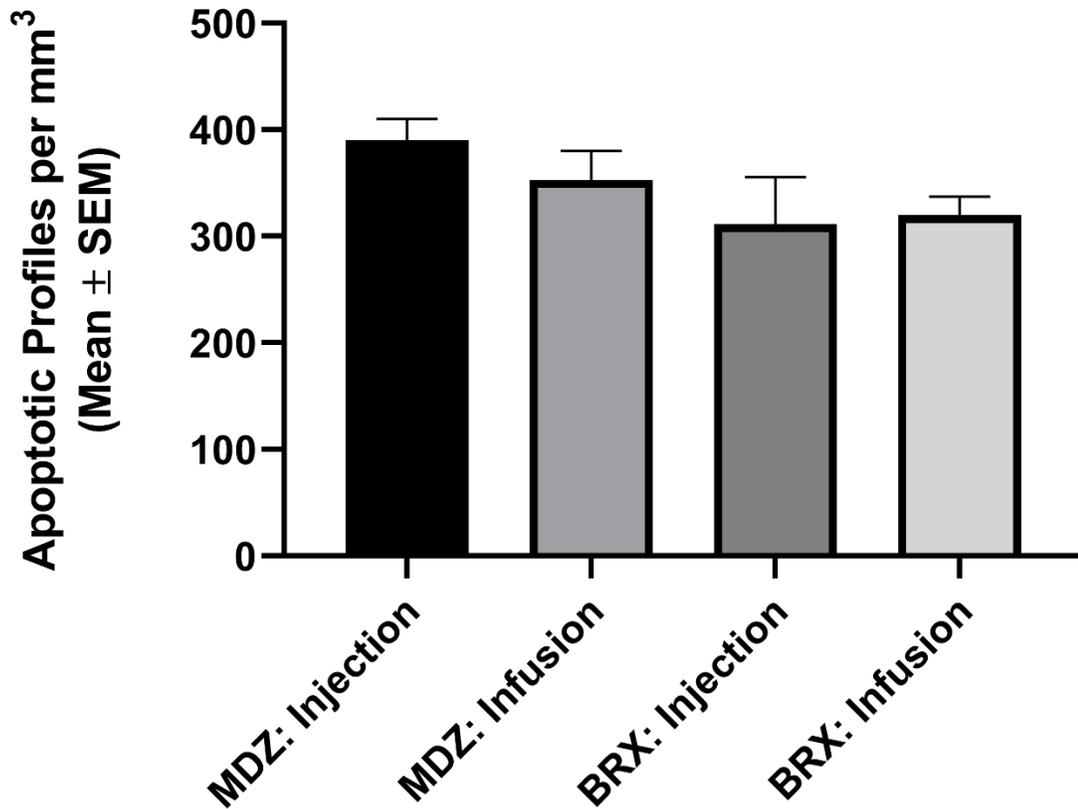


Figure 14. Mean density apoptotic profiles per mm<sup>3</sup> for animals treated with 80mg/kg BRX or 120mg/kg MDZ as either single injection or 6hr continuous infusion. Original data are presented in Figures 5, 6, and 10. Despite marginally higher apoptotic profiles for MDZ treated animals, results revealed no significant differences between all treatment groups. These data indicate BRX can induce a similar level of neurotoxicity as MDZ when administered at high doses. Importantly, the neurotoxic effects of both BRX and MDZ plateaued by 40 and 60mg/kg, respectively. This suggests the neurotoxic capabilities of both drugs are limited by the dose of a single exposure, regardless of duration, when administered by acute IP injection or 6hr continuous IP infusion.

## Discussion

The present study was designed to explore the safety and efficacy of AlloP, specifically its BRX formulation, as a SAD. My pilot observations indicated BRX was able to induce deep levels of sedation, but not anesthesia, when injected IP. MDZ was comparatively examined as a SAD of the BZD class commonly used for procedural sedation in neonatal and pediatric medicine. Experiment 1 investigated the effects of BRX and MDZ after acute administration over a range of low to high doses. Experiment 2 was designed to determine the impact of BRX and MDZ during prolonged continuous infusion. The primary goal of each experiment was to investigate the sedative ability, impact to vitals, and apoptogenicity of each drug.

In experiment 1, lower doses of BRX, 10 and 20mg/kg, produced moderate-to-deep sedation that was near recovered within 3 hours. Higher doses of BRX, 40 and 80mg/kg, generated a substantial sedative effect for the duration of experimental testing, demonstrating sedation was attainable across the full range of BRX doses. In contrast, MDZ struggled to reliably induce moderate levels of sedation until the highest dose of 120mg/kg. The result appeared to diminish more slowly despite a less pronounced initial sedative effect. Figure 11C further supports this, showing the 60 and 120mg/kg MDZ had roughly equal sedative effects compared to 10 and 20mg/kg BRX. Interestingly, 30mg/kg MDZ showed the most sedative effect within the first 30min.

The ED<sub>50</sub> for hypnotic effects of MDZ was previously reported as approximately 43.5mg/kg in adult mice following a single IV injection (Ben-Shlomo et al., 2001). Here, I show the ED<sub>50</sub> for sedation of MDZ was 27.1mg/kg in PND7 mouse pups injected IP. Differences in age, route of drug administration, and behavioral evaluation likely explain

the discrepancy. When examined alongside the ED<sub>50</sub> of BRX's sedative effect, 4.7mg/kg, it is clear BRX can produce substantial sedation at much lower doses than MDZ. This is illustrated in Figure 1 indicating only the highest doses of MDZ generated mild-to-moderate sedation within the first 30min of a single injection. All doses of BRX had moderate-to-deep sedative effects.

Pulse oximetry measures were examined to ensure the sedative ability of BRX did not similarly impact vitals. Physiological measures were clearly impacted in the 80mg/kg BRX group. Hypoxemia was induced by 80mg/kg BRX within 30min post-injection. Heart and breath rate were similarly impaired 30min after injection of 40 and 80mg/kg BRX, whereas 80mg/kg alone caused a noticeable drop in pulse distention. However, lower doses of BRX at 10 and 20mg/kg were able to effectively induce moderate levels of sedation with little influence on oxygen saturation and pulse distention measures. Heart and breath rate were reduced by 20mg/kg BRX similar to that of the higher doses, albeit with faster recovery. Breath rate was the only notable measure weakened by 10mg/kg BRX which was rapidly recovered after one hour. This indicates low impact moderate levels of sedation can be obtained with low doses of BRX.

Although hypoxemia was not induced by any MDZ dose, 60 and 120mg/kg rapidly reduced SpO<sub>2</sub> to around 92% and 91%, respectively. There appeared to be no obvious dose-dependent effect for MDZ on vitals. As expected, every dose weakened heart and breath rate. Interestingly, MDZ caused a rapid spike in pulse distention, suggesting an acute but temporary elevation in cardiac output. The recovery from BRX at lower doses was more apparent than MDZ, which appeared relatively sustained over three hours. An interesting trend taken from the pulse oximetry data is that the extent of

weakened vitals was reached by about 30min to 1hr after injection of BRX, but 5 and 30min for MDZ, indicating the effects of MDZ may be faster acting than BRX in the neonatal mouse when injected IP.

The capacity to induce moderate-to-deep levels of sedation was attainable with the lower doses of BRX. The extent of weakened vital signs was marginally different between drugs until the highest BRX dose. As such, neuroapoptosis profiles were evaluated to determine whether the sedative ability of BRX translated to elevated neurotoxicity. Physiological neuroapoptosis profiles were similar to those recently reported in PND7 mice (Maloney et al., 2019; Montana et al., 2018). Animals in the 10 and 20mg/kg BRX groups had apoptotic profiles comparable to controls, but less neuroapoptosis compared to higher doses of 40 and 80mg/kg. The ED<sub>50</sub> for neurotoxicity of BRX was approximately 23.3mg/kg. In contrast, MDZ at 30–120mg/kg yielded a greater neurotoxic effect compared to controls, showing even mildly sedative doses of MDZ resulted in significant neuroapoptosis in the developing rodent brain. The ED<sub>50</sub> for neurotoxicity of MDZ was approximately 15.7mg/kg. MDZ has a rather poor sedative effect at doses lower than 30mg/kg and neurotoxicity rapidly elevates beyond 15mg/kg. As such, sedation with MDZ is unlikely to be achieved without also producing a considerable neurotoxic response.

Taken together with the dose-response data for sedation, the therapeutic range of BRX was easily attainable without grossly impacting vitals or inducing neurotoxicity. Doses of 60 and 120mg/kg MDZ were identified as equally sedative compared to 10 and 20mg/kg BRX, respectively (Figure 11C). Evaluation of neurotoxic profiles (Figure 12)

further supports dose-response results, indicating BRX produces considerably less neuroapoptosis than equally sedative doses of MDZ.

In experiment 2, sedation was determined by quantifying general motor activity during continuous infusion. We have recently reported a paradigm to maintain stable sedation via continuous IP infusion using MDZ (Noguchi et al., 2019). At doses of 20mg/kg bolus + 10mg/kg/hr BRX, I was able to create a near equal state of sedation as MDZ by four to five hours, as well as no between-group difference detectable throughout the experimental period. Neither drug produced hypoxemia. Heart and breath rate were both weakened by BRX and MDZ relative to controls. Although not statistically significant, Figure 4B indicates MDZ had a clinically relevant impact on heart rate.

Healthy human neonates have an average heart rate of approximately  $122.18 \pm 11.43$  BPM during the first week of life, with a minimum lowest value of about 92 BPM (Longin et al., 2005). Bradycardia is defined as a decrease in heart rate below an established lower limit (Baruteau et al., 2016). Thus, a 31% decrease in heart rate as shown in the MDZ group would reduce a neonate's BPM to approximately  $83.91 \pm 7.85$ . This is well within the range of bradycardia and increases the possibility of additional medical complications and sudden death. On the other hand, BRX induced a decline in heart rate of  $100.87 \pm 9.44$  BPM.

Similar but less robust effects were also observed with breath rate. The BRX group had a stabilized breath rate around two hours after the start of infusion but was higher compared to MDZ treated animals. Respiratory depression is difficult to diagnose because of the variability of normal breath rate in neonates (Reuter et al., 2014). With that said, both drugs showed repressed breath rate over time compared to controls.

While translation from preclinical data to clinical practice is difficult, these results suggest BRX might be less deleterious to heart and breath rate compared to MDZ during prolonged infusion. Indeed, continuous infusions with MDZ is cautioned as it poses risks of hypotension, respiratory depression, and hypoxemia (FDA 2017; Ng et al., 2017; Durrmeyer et al., 2010). Accumulation of MDZ's active metabolite,  $\alpha$ -hydroxy-midazolam, in peripheral tissue makes dosing difficult and requires the lowest possible doses of MDZ to be administered to prevent physiological complications. This makes MDZ difficult to administer beyond lesser sedative doses without adjunct treatment of additional SADs, several of which have been shown to exacerbate neurotoxicity (Jevtovic-Todorovic et al., 2003; Young et al., 2005).

Caffeine is also commonly given to attenuate respiratory depression and prevent apnea in preterm neonates. Adjunct treatment with caffeine has been shown to dramatically increase neuroapoptosis and produce long-term behavioral deficits when coupled with SADs, including MDZ (Cabrera et al., 2017; Yuede et al., 2013). While no neurotoxic data have been published on the effects of combining caffeine with AlloP, some findings point to a negative impact on respiratory function (Uppari et al., 2016). AlloP reduced respiratory function and increased the frequency of apnea when combined with caffeine. Although the dose of caffeine (15mg/kg oral) was considerably less than previously examined (80mg/kg IP; Cabrera et al., 2017; Yuede et al., 2013), AlloP at 10mg/kg did not alter respiratory function in no-caffeine controls at PND12 (Uppari et al., 2016), supporting results presented here. These data lend credence to the usefulness of AlloP's acute application, as detailed in this study, considering adjunct therapy with caffeine during continuous infusion might not be possible with AlloP.

Prolonged infusion of BRX and MDZ produced relatively similar apoptotic profiles per  $\text{mm}^3$ . This is interesting given results from lower dose, single injections of BRX. Neither drug induced hypoxemia in experiment 2, nor did the effects on other vitals appear to influence neurotoxic profiles in any sensible manner. The logical assumption is a dose-dependent threshold of neurotoxicity in which the neurotoxic effects of BRX are not produced until high doses. This is in line with the neurotoxic profiles from single injections presented in Figure 5. Doses of 80mg/kg BRX and 120mg/kg MDZ were the same quantity of drug administered during continuous infusion. Independent examination of neurotoxic profiles from 80mg/kg BRX and 120mg/kg MDZ compared to results from experiment 2 yielded no significant differences (Figure 14). Indeed, BRX treated animals showed near identical mean apoptotic profiles per  $\text{mm}^3$  from 80mg/kg given as a single injection or continuous infusion.

Although this explanation disregards pharmacokinetic contributions as drug effect and metabolism would be different between single and prolonged exposure, it may explain why BRX delivered similar neuroapoptosis compared to MDZ during prolonged infusion. Importantly, it is unknown whether a specific regimen of AlloP could be delivered to maintain chronic sedation with low apoptogenicity. Figure 14 suggests the neurotoxicity of BRX and MDZ might be dependent on the dose rather than the duration of a single exposure.

Another possibility includes a neurotoxic ceiling in which the apoptogenic potential is exhausted regardless of dose. Indeed, the neurotoxic effects of BRX plateaued by a single injection of 40mg/kg. This suggests the neurotoxic threshold of BRX lies somewhere between 20 and 40mg/kg, and 15 and 30mg/kg for MDZ. This also points to

the possibility that transient exposure to BRX and MDZ has limited apoptogenicity, although the possibility of a neurotoxic ceiling is supported more considering 40mg/kg BRX induced as much neurotoxicity as 80mg/kg BRX. Taking into consideration data in Figure 14, this would point to the unlikely conclusion that continuous infusion is less neuroapoptotic than acute exposure of high doses of BRX.

These findings bring into question why low doses of BRX were able to induce transient moderate-to-deep sedation with minimal neurotoxicity. One explanation could be that low doses of BRX enact neuroprotection against GABA-induced apoptosis in the developing brain. Several lines of research have focused intently on understanding the neuroprotective and regenerative capabilities of AlloP in a variety of diseases (Irwin & Brinton, 2014). To my knowledge, no studies have yet investigated the neurotoxic or neuroprotective capabilities of AlloP in the context of SAD-induced neuroapoptosis.

Most existing research in neonates or *in utero* focuses on AlloP as a neuroprotectant to hypoxia-ischemia. For instance, Tsuji and colleagues (2012) used a hypoxia-ischemia model to test whether P4 or AlloP were neuroprotective in the developing rat brain, as they are in adults. They found P4 and AlloP dose- and age-dependently increased damage by measures of reduced ipsilateral hemispheric volumes in the cortex, striatum, hippocampus, and thalamus. P4 and AlloP exacerbated hypoxia-ischemia damage in the PND7 rat, to a lesser extent in the PND14 compared to PND7 rat and showed no significant differences in the PND21 group. Co-administration with bicuculline, a GABA<sub>A</sub>R antagonist, attenuated reductions in hemispheric volume in the PND7 group by qualitative evaluation. The authors suggest the GABAergic activity of AlloP potentiated hypoxia-ischemia (Tsuji et al., 2012).

At face value the age-dependent response from Tsuji et al (2012) aligns with the window of vulnerability for SAD-induced neuroapoptosis and could explain why GABA<sub>A</sub> activation via AlloP did not continue to worsen hypoxia-ischemia damage by PND21 (Maloney et al., 2018). One problem with this interpretation is that animals were not sacrificed until seven days after hypoxia-ischemia and the residual neurotoxic effects from low doses of AlloP (10 mg/kg) would not likely be detectable by volumetric changes. Results presented here support this position with no neurotoxic response different from controls with 10mg/kg. One explanation may be an effect of AlloP that potentiated hypoxia-ischemia by mechanisms different than those of traditional SAD-induced neurotoxicity. Importantly, no differences were detected in contralateral, i.e. hypoxia-ischemia control, hemispheric volumes within and between groups, indicating AlloP administration alone did not reduce volumetric measures (Tsuji et al., 2012). Indeed, some SADs have shown notable impacts on brain volume in rodents and humans (Duerden et al., 2016; Ikonomidou et al., 2007; Liu et al., 2002).

The benefits reported from coadministration of AlloP and bicuculline entertains the possibility that AlloP has neurotoxic effects in the developing rodent brain, possibly similar to other GABAergic SADs. However, the authors could only distinguish qualitative differences and, when considering results from Figure 5, the apoptogenicity of AlloP is only detectable at doses much higher than 10mg/kg. Thus, AlloP might hold GABAergic properties distinct from other SADs and protective factors may be afforded only under certain physiological conditions or at specific doses in the developing brain.

Finally, Tsuji et al (2012) injected AlloP at a dose previously shown to produce cortical AlloP levels similar to rat fetuses during late gestation (~20 ng/g), but this dose

was determined from AlloP concentrations found in adult mice (22 ng/g) with no evidence of similar serum or brain AlloP levels in neonates (Wang et al., 2010). Indeed, PND5 rats injected with 20mg/kg AlloP had hippocampal concentrations of  $322.45 \pm 76.43$  ng/g (Mòdol et al., 2013). Considering the present findings, AlloP at this dose does not produce neurotoxicity in PND7 mice and the lack of brain AlloP data make it difficult to conclude the GABAergic properties of AlloP directly potentiated hypoxia-ischemia damage by mechanisms relevant to SAD-induced neurotoxicity (Tsuji et al., 2012).

This is more apparent when considering research in fetal sheep showing stimulation of endogenous AlloP production in the brain, but not periphery, reduces hypoxic damage (Hirst et al., 2008). The authors propose AlloP elicits an endogenous protective mechanism following hypoxia. Interestingly, suppression of AlloP synthesis using the  $5\alpha$ -R inhibitor, finasteride, provoked extensive neurotoxicity in the fetal sheep brain, identified by apoptotic cells (Yawno et al., 2007; Yawno et al., 2009). In some brain regions the apoptotic response consequent to AlloP deprivation was greater than the hypoxia-only condition, indicating AlloP plays a key role in regulating physiological neuroapoptosis in the developing brain (Yawno et al., 2009). Taken together with results from the current study, additional research is needed to investigate whether AlloP has direct neuroprotectivity against SAD-induced neurotoxicity in the developing rodent brain.

The present findings also show a possible contribution of the BRX vehicle, captisol. Data in Figure 13 show captisol at  $7.5 \mu\text{l/g}$  reduced physiological neuroapoptosis compared to saline controls. This is interesting considering a hydroxypropyl- $\beta$ -

cyclodextrin has been shown to reduce hypoxia-ischemia in young rats (Rivers et al., 2012). Additionally, the neuroprotective effect of AlloP in neonatal models of NP-C were later attributed to the hydroxypropyl- $\beta$ -cyclodextrin vehicle (Griffin et al., 2004; Ottinger et al., 2014). To my knowledge, no data have been published on any neuroprotective effect of captisol, a sulfobutylether- $\beta$ -cyclodextrin, in young or neonatal rodents. However, prior findings with hydroxypropyl- $\beta$ -cyclodextrins should be considered when interpreting the effects of BRX.

Another reason AlloP might be less neuroapoptotic may not necessarily be neuroprotection, but rather that neurosteroids are simply less apoptogenic in the developing brain compared to SADs. Maternal steroidal support for the fetus is crucial to many essential stages of neurodevelopment. Preterm infants are susceptible to a drastic and early withdrawal from maternally derived neurosteroids compared to full-term births. This can leave the brain particularly vulnerable to excitotoxicity (Hirst et al., 2009). Reduced myelination is also a key area of focus as the deprivation of AlloP may prevent steady proliferation of oligodendrocytes and stable formation of subcortical white matter. Studies in preterm infants and various animal models have associated SADs with reductions in white matter density and subsequent long-term behavioral consequences (Brunton et al., 2014; Shaw et al., 2019a). As such, the supplementation of neurosteroids in the preterm infant has been proposed for AlloP and AlloP-analogs (Brunton et al., 2014; Shaw et al., 2019a).

The short half-life of AlloP would make it difficult to use as supplementation following deprivation of maternal neurosteroids (Shaw et al., 2019a). The proposed use of AlloP analogs is growing with recent data demonstrating ganaxolone attenuates poor

myelination in a guinea pig model of preterm birth (Shaw et al., 2019b). Ganaxolone has recently been reported as a neuroprotective treatment for neonatal seizures, safeguarding against the excitotoxic effects of hypoxia-ischemia (Yawno et al., 2017). However, these results are not surprising as AlloP is involved with regulating and/or protecting against hypoxia-induced apoptosis in the developing sheep brain (Yawno et al., 2007). Whether or not this is protective in the context of SAD-induced neurotoxicity remains unknown.

Another AlloP analog, alfaxalone, can induce acute anesthesia with no significant neurotoxic response in the neonatal mouse (Montana et al., 2018). The sedative effect of alfaxalone was comparable to BRX in that 30mg/kg IP induced loss of righting ability for  $89\pm 41$ min. Findings with alfaxalone are similar to the present results in which acute, deep levels of sedation can be delivered with minimal neurotoxicity using BRX. Recent data from Tesic and colleagues (2020) support these findings in which alfaxalone and CDNC24, another GABAergic neurosteroid, produced anesthesia and had a higher therapeutic index compared to propofol. Both drugs also produced no significant increase in neurotoxicity in the medial prefrontal cortex and subiculum in PND7 rats (Tesic et al., 2020).

Continuous IV infusion of alfaxalone at 5mg/kg/hr ameliorated the impacts of finasteride-induced cell death, identified by pyknotic cells, and decreased cellular proliferation in the fetal sheep (Yawno et al., 2009). Importantly, continuous infusion of alfaxalone alone did not induce apoptotic cell death in the fetal brain. Blocking conversion of  $5\alpha$ -reduced AlloP has been shown to induce fetal neurotoxicity (Yawno et al., 2007), and alfaxalone was able to rescue these effects when co-infused with

finasteride (Yawno et al., 2009). This points to an exciting possibility that AlloP and AlloP-analogs might serve as non-apoptogenic GABA<sub>A</sub> agonists in the developing brain.

These findings bring into question the potential neurotoxic effects of neurosteroid supplementation in preterm neonates. Ganaxolone showed sedation at doses of 2.5mg/kg delivered twice daily until the term equivalence, implying activity at the levels of the CNS (Shaw et al., 2019b). Results from the present study and others suggest sedation and anesthesia can be delivered using non-neurotoxic doses of BRX and alfaxalone (Montana et al., 2018; Tesic et al., 2020). Despite these observations, there is not enough data to systematically conclude the maximum effective doses and number of exposures of these neurosteroids deliverable without also inducing neurotoxicity. The matter is likely context specific and will be limited to experimental goals. This is demonstrated by the present infusion data in which prolonged light sedation with BRX was not only similarly neurotoxic compared to MDZ, but also to a single injection of the same dose. As such, careful consideration should be taken when dosing AlloP for use as a neurosteroid supplement or as a SAD. Drug, route of administration, metabolism, and clearance of active metabolites will certainly contribute to neurotoxic thresholds.

Additionally, the consequences of neurotoxicity vs. other neuroprotective and neuroregenerative effects should be weighed. For example, neurosteroid supplementation of ganaxolone twice daily at 2.5mg/kg SC improved subcortical white matter formation but also induced lethargy and weight loss, leading to higher mortality rates (Shaw et al., 2019b). Alfaxalone prevented finasteride-induced neurotoxicity and reduced cellular proliferation. However, minimal differences were detected compared to controls, drawing

concern of its usefulness at low doses outside of hypoxia-ischemia models (Yawno et al., 2009).

The protective effects of neurosteroid supplementation should be examined comparatively using detailed drug administration paradigms. For instance, the benefits from preterm AlloP supplementation might outweigh the consequences of neurotoxicity, assuming proapoptotic doses are needed. This would lend support to supplementing AlloP over other SADs when continuous infusions are required. Neonates might also benefit from AlloP following acute exposure, but caution should be taken even with non-neurotoxic doses. Research with rodents indicates doses as low as 10 and 20mg/kg AlloP during early postnatal periods can induce abnormal behavioral alterations detectable into adulthood, including long-term anxiety, stunted aversion learning, decreased prepulse inhibition, and lasting hyperactive motor function (Grobin et al., 2006; Mòdol et al., 2013). Additionally, neonatal exposure to supraphysiological levels of AlloP may stunt endogenous neurosteroid synthesis in adulthood (Grobin et al., 2006). As such, detailed investigation of long-term behavioral and neurophysiological consequences of neonatal exposure to AlloP should be established in a context tailored toward clinical translation.

### **Conclusion**

Results from the present study support an emerging interest in using neurosteroids as SADs. The GABAergic properties of neurosteroids such as AlloP have long been known to produce CNS depression. Several lines of research now support the neuroprotective and/or non-apoptogenic nature of these drugs, but studies in neonatal rodents are sparse. Here, I report sedative doses of BRX can be administered acutely as a non-neurotoxic alternative to MDZ with low impact to physiological vitals in neonatal

mice. The large therapeutic range of AlloP permits transient mild to deep sedation without neurotoxic consequences. Comparison of equally sedative doses of BRX and MDZ further supports the idea that a single sedative injection of the neurosteroid BRX is less apoptogenic than a commonly used SAD. On the other hand, prolonged light sedation with BRX in a 6hr continuous infusion paradigm delivered similar neurotoxic profiles as MDZ. Supplementary analysis revealed this effect was likely driven by total dose and not the duration of exposure, but also implies sustained non-neurotoxic sedation using BRX may not be possible. Taken together with the dose-response data, a neurotoxic threshold between 20 and 40mg/kg likely exists for BRX. Further research is required to determine optimal rate of exposure and proper doses of AlloP to induce prolonged sedation without neurotoxic consequences.

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