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Potential Neurodevelopmental Effects of Pediatric Intensive Care Sedation and Analgesia: Repetitive Benzodiazepine and Opioid Exposure Alters Expression of Glial and Synaptic Proteins in Juvenile Rats

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Objectives: Sedatives are suspected contributors to neurologic dysfunction in PICU patients, to whom they are administered during sensitive neurodevelopment. Relevant preclinical modeling has largely used comparatively brief anesthesia in infant age-appropriate animals, with insufficient study of repetitive combined drug administration during childhood. We hypothesized that childhood neurodevelopment is selectively vulnerable to repeated treatment with benzodiazepine and opioid. We report a preclinical model of combined midazolam and morphine in early childhood age-appropriate rats.

Design: Animal model.

Setting: Basic science laboratory.

Subjects: Male and female Long-Evans rats.

Interventions: Injections of morphine + midazolam were administered twice daily from postnatal days 18–22, tapering on postnatal days 23 and 24. Control groups included saline, morphine, or midazolam. To

screen for acute neurodevelopmental effects, brain homogenates were analyzed by western blot for synaptophysin, drebrin, glial fibrillary acidic protein, S100 calcium-binding protein B, ionized calcium-binding adaptor molecule 1, and myelin basic proteins. Data analysis used Kruskal-Wallis with Dunn posttest, with a p value of less than 0.05 significance.

Measurements and Main Results: Morphine + midazolam and morphine animals gained less weight than saline or midazolam ($p \leq 0.01$). Compared with saline, morphine + midazolam expressed significantly higher drebrin levels ($p = 0.01$), with numerically but not statistically decreased glial fibrillary acidic protein. Similarly, morphine animals exhibited less glial fibrillary acidic protein and more S100 calcium-binding protein B and synaptophysin. Midazolam animals expressed significantly more S100 calcium-binding protein B ($p < 0.001$) and 17–18.5 kDa myelin basic protein splicing isoform ($p = 0.01$), with numerically increased synaptophysin, ionized calcium-binding adaptor molecule 1, and 21.5 kDa myelin basic protein, and decreased glial fibrillary acidic protein.

Conclusions: Analysis of brain tissue in this novel rodent model of repetitive morphine and midazolam administration showed effects on synaptic, astrocytic, microglial, and myelin proteins. These findings warrant further investigation because they may have implications for critically ill children requiring sedation and analgesia.

Key Words: benzodiazepine; glial and synaptic proteins; neurodevelopment; opioid; pediatric intensive care; sedative neurotoxicity

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Neurodevelopmental consequences of sedatives and analgesics that alter neurotransmission by their very design are not entirely understood. However, there are compelling preclinical animal model data supporting deleterious effects on the neonatal and infant mammalian brain after relatively brief exposures to common surgical anesthetics (1, 2). Despite this evidence,

there is little to no investigation representing the sustained administration of sedatives and analgesics to infants, children, and adolescents in the PICU setting (3–5). Although anesthesia is an almost uniformly singular event, survivors of childhood critical illness are exposed to similarly neuroactive sedatives and analgesics in a variety of combinations, frequently as continuous infusions, in escalating doses, over days to weeks exclusive of additional weaning to abstinence during convalescence and recovery.

Seminal work in this area began with episodic exposures to inhalational anesthetics that both block *N*-methyl-D-aspartate receptors and promote γ -aminobutyric acid (GABA) signaling, and similar findings were also reported for parenteral propofol, ketamine, and benzodiazepines (6–13). Those studies in rodents and non-human primates reproducibly demonstrated increased neuronal death and reduced synaptic growth and refinement. However, a timeline for neurodevelopmental vulnerability still needs to be delineated because the bulk of those studies were performed in neonate and infant age-approximate rodents from birth to postnatal day (PND) 14, a time period coinciding with peak dendritic spine formation and synaptogenesis before transition to early childhood at PND 15, and also preceding the peak of brain myelination (14–16). Interestingly, Briner et al (17) showed suppression of dendritic synaptogenesis with 6-hour sedation with either propofol or midazolam in PNDs 5 and 10 rats but described an opposite effect of increased dendritic synaptogenesis in childhood age-approximate rats at PNDs 15, 20, and 30 (18). These effects were long lasting and persisted after adulthood was reached at PND 60. No effect was found after PND 60 or 90 exposure, indicating at least two distinctly sensitive epochs in brain development, and recommending similar agents for investigation across a broader age range.

Importantly, there are age-dependent variations in the balance and coordination of neurodevelopmental processes, and, thus, it is logical to hypothesize that there will be age-dependent variation in the neuroactive effects of sedatives and analgesics. Although human synaptogenesis peaks in the first 2 years, peak myelination, synaptic remodeling, selective neuronal apoptosis, and ongoing neurogenesis and glial cell generation are equally critical at later stages of brain maturation. These crucial mechanisms confer the plasticity necessary for increasingly complex cognition and environmental interactions. Furthermore, they are also vital in maintaining neurologic integrity after a variety of insults and stressors, from primary brain injuries to critical illness hypoxemia, ischemia, inflammation, and oxidative stress.

It must be noted that despite reproducible laboratory evidence of anesthetic neurotoxicity, studies in human children have been less uniform. Although some studies observed lower age-matched academic achievement test scores and more learning disabilities after elective surgical anesthesia in young children, more recent prospective landmark clinical trials detected no impact on cognitive function or behavior (19–24). Although this dichotomy might also apply to PICU-typical sedation in an otherwise healthy child, critically ill children are not otherwise healthy. Critical illness is a milieu of inflammation, metabolic disturbance, and oxidative stress often in the midst of hypoxemia and ischemia. Clinical studies have identified neurologic morbidity after childhood critical

illness such as delirium, posttraumatic stress, cognitive and functional decline, and need for neurologic evaluation (25–27). As in adults, delirium may be a harbinger of children at risk for neurologic morbidity, and this link was recently reported in a study of over 2,000 PICU patients that found the following to be associated with delirium: age less than 2 years, mechanical ventilation days, higher-dose benzodiazepine, and low or moderate dexmedetomidine exposure, among other sedation and illness factors (28, 29). Interestingly, fentanyl as the primary opiate, days of propofol, depth and days of sedation, inadequate pain control, and days of agitation were also associated with delirium. Given clinical evidence of this complex problem, there is a need to better understand the interplay between the neurologic stressors of critical illness and routinely administered sedatives and analgesics.

To address a critical lack of preclinical models of PICU-typical sedation, we report here a novel juvenile rat model of 7-day combined morphine and midazolam treatment in a rodent age more consistent with early childhood than robustly reported neonate and infant age-approximate models. We hypothesized that an early childhood neurodevelopmental time point is selectively vulnerable to prolonged, repeated administration of combined opioid and benzodiazepine already commonplace in contemporary pediatric intensive care. This model is feasible and accessible without the need for continuous infusion, intraperitoneal injections, or invasive mechanical ventilatory support. Importantly, it provides a safe and efficacious initial dose range for future study, particularly in combined models of PICU-typical illnesses and injuries where animal viability is a concern.

MATERIALS AND METHODS

Animal Breeding and Housing Procedures

Experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals in Research and under protocols approved by the Animal Care and Use Committee of Virginia Commonwealth University. Outbred Long-Evans male and female rats were the offspring from timed pregnant dams provided by Charles River Laboratories (Raleigh, NC). Dams were obtained early on gestational day 2 to minimize potential shipping stress effects on prenatal brain development. Litters were group housed with their own natural mother throughout experiments until the end of study, in ventilated 43 cm × 30 cm × 19 cm clear polycarbonate cages under controlled temperature, with ad libitum chow and water, 12-hour regular light/dark cycles, and wood chew and cardboard tube enrichment. All experimental animals had equal access to nursing and chow, with equal durations of maternal separation during drug or saline administration.

Study Design

On PND 18, animals within each litter were randomly divided into the different experimental groups: morphine + midazolam (Morph/Midaz; $n = 13$), morphine (Morph; $n = 12$), midazolam (Midaz; $n = 12$), and saline (Sal; $n = 10$). Male and female breakdown in each experimental group was as follows: Morph/Midaz (7 male, 6 female), Morph (7 male, 5 female), Midaz (8 male, 4 female), and

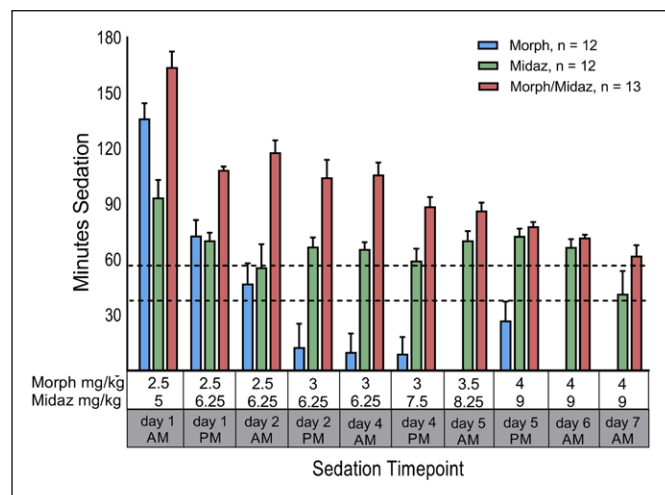


Figure 1. Average sedation duration and drug dose, postnatal days 18–24 rats underwent bid sedations for 5 d using morphine + midazolam (Morph/Midaz), morphine (Morph), midazolam (Midaz), or saline (Sal). Dosing was titrated for 60- to 90-min sedation after Morph/Midaz treatment. As anticipated, both Morph and Midaz treatments resulted in shorter duration, or no sedation, over time. Sal control animals did not lose righting reflex and are not graphically represented. The required dosage is noted for each study timepoint. Data are depicted as mean and SEM.

Sal (5 male, 5 female). As outlined in **Figure 1**, escalating doses of Morph/Midaz (dose range = 2.5–4 mg/kg Morph and 5–9 mg/kg Midaz) were administered to produce sedation as defined by the absence of righting reflex without apnea. Because drug metabolism in rodents is faster than in humans, higher relative doses are required for equipotent effect given size and body surface area (30–32). We, therefore, targeted a clinically relevant 60- to 90-minute sedation duration representing a typical effect in children after one-time administration of combined opioid and benzodiazepine. To establish the doses to achieve this effect, we carried out pilot studies that considered some of the previously reported concentrations used by others in different animal models (4–6, 18, 22, 23). Study drugs (or no drug in Sal) were diluted in 0.9% saline for a uniform volume of 5 mL/kg calculated with an online veterinary calculator (VPRcloud.com), and administered subcutaneously by a blinded investigator at the nape of the neck with a 30 gauge needle (Nipro, Bridgewater, NJ). Animals were dosed twice daily for 5 days (PNDs, 18–22), followed by once daily weaning doses with no sedative effect on PNDs 23 and 24. Sedations were performed at 08:00 AM in the light cycle, and 16:00 PM for recovery during the dark cycle. Supplemental oxygen was titrated for oxyhemoglobin saturations (oxyHb%) greater than or equal to 92%, and F_{IO_2} ranged between 0.29 and 0.42 with a mean of 0.36. Animals were noninvasively monitored during sedation in a 34–38°C warmed container for oxyHb% and heart rate using medium collar clips and consecutive autocycling every 5 seconds (MouseOx Plus with Multiplexer; STARR Life Sciences, Oakmont, PA). Darkly hooded Long-Evans rats required shaving of fur from the neck on study day 1. Animals later identified as saline and single-drug controls were frequently too arousable to cooperate with a collar, and thus, uncooperative animals with regular respirations and pink noses and paw pads were assumed to have adequate oxyHb%. On PND 24, rats were anesthetized with isoflurane and killed by transcardial perfusion with ice-cold phosphate-buffered

saline (PBS). Brains were removed and snap frozen and stored at -80°C for western blot analysis.

Western Blot Protocol

Brain tissue was homogenized in PBS with Halt Protease Inhibitor Cocktail with EDTA (Thermo Fisher, Waltham, MA) before solubilization in 2. Laemmli buffer and 5% β -mercaptoethanol. Tissue protein content for each sample was determined by the Bradford method. Protein separation used sodium dodecyl sulfate electrophoresis in TGX Stain-Free 4–15% acrylamide gels followed by 45-second ultraviolet light exposure to activate tryptophan residues for total protein band quantification (Bio-Rad, Hercules, CA). Protein electrotransfer to nitrocellulose membranes was carried out with Bio-Rad Laboratories equipment and reagents (Bio-Rad). Protein electrotransfer to nitrocellulose membranes was carried out with Bio-Rad Laboratories equipment and reagents (Bio-Rad, Hercules, CA). Western blotting used the following primary antibodies: anti-myelin basic protein (MBP; dilution 1:500; MilliporeSigma, Burlington, MA), anti-synaptophysin (SYN; 1:1,000; Cell Signaling, Beverly, MA), anti-drebrin (1:1,000; Cell Signaling), anti-glial fibrillary acidic protein (GFAP; 1:2,000; Cell Signaling), anti-S100 calcium-binding protein B (S100B; 1:1,000; Cell Signaling), and anti-ionized calcium-binding adaptor molecule 1 (Iba1; 1:1,000; Wako, Richmond, VA). After horseradish peroxidase-conjugated secondary antibody incubation, immunoreactive bands were detected by enhanced chemiluminescence reaction with either Clarity or Clarity Max reagent and imaged with a Bio-Rad ChemiDoc MP Imager (Bio-Rad). Immunoreactive band relative expressions were determined by scanning densitometric analysis using both the ImageJ (National Institutes of Health, Bethesda, MD) and ImageLab programs (Bio-Rad). All target proteins were divided by the respective 50 kDa protein band of ultraviolet light-activated tryptophan residues to standardize protein loading and transfer conditions (33).

Statistical Analysis

Weight, oxyHb%, heart rate, F_{IO_2} , and sedation duration were recorded daily. Cerebral homogenate samples for each experimental drug condition (Morph/Midaz, Morph, and Midaz) were grouped and processed with respective Sal for comparison. To evaluate sex differences and control for sexual dimorphism in pup size, male and female samples were first analyzed separately and then combined for a larger prepubertal group analysis. Western blot semiquantitative evaluations of relative optical density were blinded and performed by two separate observers. All results were analyzed using Kruskal-Wallis with Dunn posttest with GraphPad Prism 8 software (GraphPad Software, San Diego, CA). Sedation duration data are reported and depicted as mean \pm SEM. Weight gain and western blot data are reported as median followed by interquartile range (IQR) in parentheses, with significance determined by a p value of less than 0.05. Data are graphically depicted in box and whisker plots, with whiskers indicating minimum and maximum values of all data. Individual data points are also displayed. For ease of visual interpretation of the reader, saline values were combined within graphs of each protein of interest because there were no significant differences between gels.

RESULTS

Model Development

Previous studies reported the use of PND 0–30 pups and adult rodents with doses of 10 mg/kg morphine and 9–40 mg/kg midazolam (4–6, 22, 23). However, in our hands and with the present animal model, our pilot study showed that a first-dose combination of 5 mg/kg morphine + 15 mg/kg midazolam resulted in 50% mortality from apnea. Subsequent first-dose combination of Morph/Midaz (2.5 mg/kg morphine + 5 mg/kg midazolam) was reduced and well tolerated with no mortality. As shown in Figure 1, animals displayed narcotic tolerance, requiring a dose increase of either midazolam or morphine roughly every third dose to attain a 60–90 minutes of sedation induced by Morph/Midaz. Cumulative doses for 7 days were as follows: 35 mg/kg morphine and 79.5 mg/kg midazolam, for both combination and single-drugs.

Morph/Midaz animals were markedly sedated compared with all other groups and were the only group successfully monitored; average Morph/Midaz oxyHb% and heart rate are displayed in Table 1. After injections, the Morph group displayed stereotypic licking and chewing that was not only absent in the Midaz animals but also in the Morph/Midaz rats, an effect possibly related to Midaz coadministration or sedation depth. Interestingly, on study days 3–5, Midaz-treated animals adopted a side-lying C-shaped posture, and during recovery from acute drug effect manifested various degrees of disorganized “darting and zooming” behavior.

Weight gain in Morph was significantly reduced compared with both Sal and Midaz ($p < 0.0001$), and weight gain in Morph/Midaz was significantly reduced compared with Sal and Midaz ($p < 0.01$). As shown in Figure 2, median weight gain for both sexes from PND 18 to PND 24 was Sal 73.2% (IQR, 66.9–75.1) and Midaz 73.7% (IQR, 67.5–79.4), versus Morph 54.1% (IQR, 48.4–57.3) and Morph/Midaz 59.2% (IQR, 55.6–65.6). Failure to gain weight was most pronounced in Morph males compared with either Sal or Midaz (Morph 53.9% [IQR, 47.7–57.1] vs Sal 73.7% [IQR, 71.0–78.1], $p < 0.001$; vs Midaz 73.7% [IQR, 66.3–86.0],

TABLE 1. Morphine + Midazolam Physiologic Data

Timepoint	Oxyhemoglobin Saturation	Heart Rate (beats/min)
Day 1 AM	94.5 ± 1.5	418.0 ± 10.3
Day 1 PM	90.7 ± 1.5	414.5 ± 16.4
Day 2 AM	94.9 ± 2.5	419.5 ± 14.5
Day 2 PM	96.5 ± 0.9	439.6 ± 29.5
Day 3 AM	95.2 ± 1.4	444.8 ± 29.5
Day 3 PM	96.4 ± 0.5	421.4 ± 9.8
Day 4 AM	94.6 ± 1.7	423.9 ± 13.2
Day 4 PM	97.4 ± 0.6	404.2 ± 12.1
Day 5 AM	95.2 ± 1.3	407.2 ± 11.4
Day 5 PM	96.0 ± 1.7	405.4 ± 21.5

Data are expressed as mean ± SEM.

$p < 0.001$). Morph/Midaz-treated males also gained less weight than Sal or Midaz (Morph/Midaz 58.8% [IQR, 55.6–65.0], $p = 0.01$). In Morph- and Morph/Midaz-treated females, the difference in weight gain was significantly reduced only compared with Midaz (Midaz 73.7% [IQR, 69.5–76.4] vs Morph 54.3% [IQR, 48.6–61.5], $p < 0.01$; vs Morph/Midaz 61.1% [IQR, 55.6–65.7], $p = 0.04$). Despite reduced weight gain in animals that received Morph or Morph/Midaz, there were no differences in coat luster and no coat shabbiness; there was no porphyrin staining, lethargy, or anhedonia to indicate obvious impacts on animal robustness.

Assessment of Treatment Effects by Western Blot Analysis

To assess potential neurodevelopmental effects, we evaluated cerebral levels of SYN and drebrin as markers for altered dendritic synaptogenesis or plasticity (9, 10). Glial fibrillary acidic protein (GFAP) was used as a marker of astrocyte activation, S100B was used as a marker of mature astrocytes, and MBP expression was used as a reporter of mature oligodendrocytes and myelination (34). Iba1 is a microglial marker and is chosen to screen for the presence of potential brain inflammation.

Importantly, as shown in Figure 3, median S100B expression was significantly increased by Midaz treatment in prepubertal pups (Midaz 170.0% [IQR, 128.0–206.0] vs Sal 101.0% [IQR, 92.9–105], $p < 0.001$). This effect was similar in males and females (male 176.0% [IQR, 131.0–206.0], $p = 0.01$; female 152.0% [IQR, 125.0–239.0], $p = 0.05$). Treatment with Morph numerically increased median S100B without a male-female difference (124.0% [IQR, 98.4–143.0] vs Sal 98.0% [IQR, 94.0–110.0], $p = 0.13$), whereas combined treatment with Morph/Midaz did not significantly change S100B (82.3% [IQR, 56.4–104.0] vs Sal 97.4% [IQR, 88.7–117], $p = 0.29$). Also shown in Figure 3 is GFAP expression, which was reduced by more than 20% in all drug treatment groups in prepubertal pups

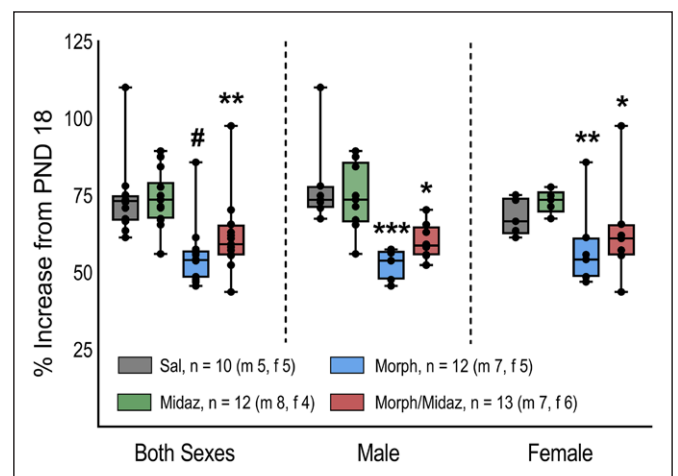


Figure 2. Percentage weight gain from postnatal day (PND) 18 to PND 24. Weight gain was calculated as percentage of PND 18 baseline weight gained by study day 7 on PND 24. There were no differences between any saline (Sal) and midazolam (Midaz)-treated groups. Median percentage weight gain in both sexes morphine (Morph) and morphine + midazolam (Morph/Midaz) groups was lower than percentage weight gain in both sexes Midaz and Sal groups. This pattern is seen in males, but in Morph and Morph/Midaz females, this difference is significant only in Morph and Morph/Midaz versus Midaz. Using Sal or Midaz as a reference: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.0001$.

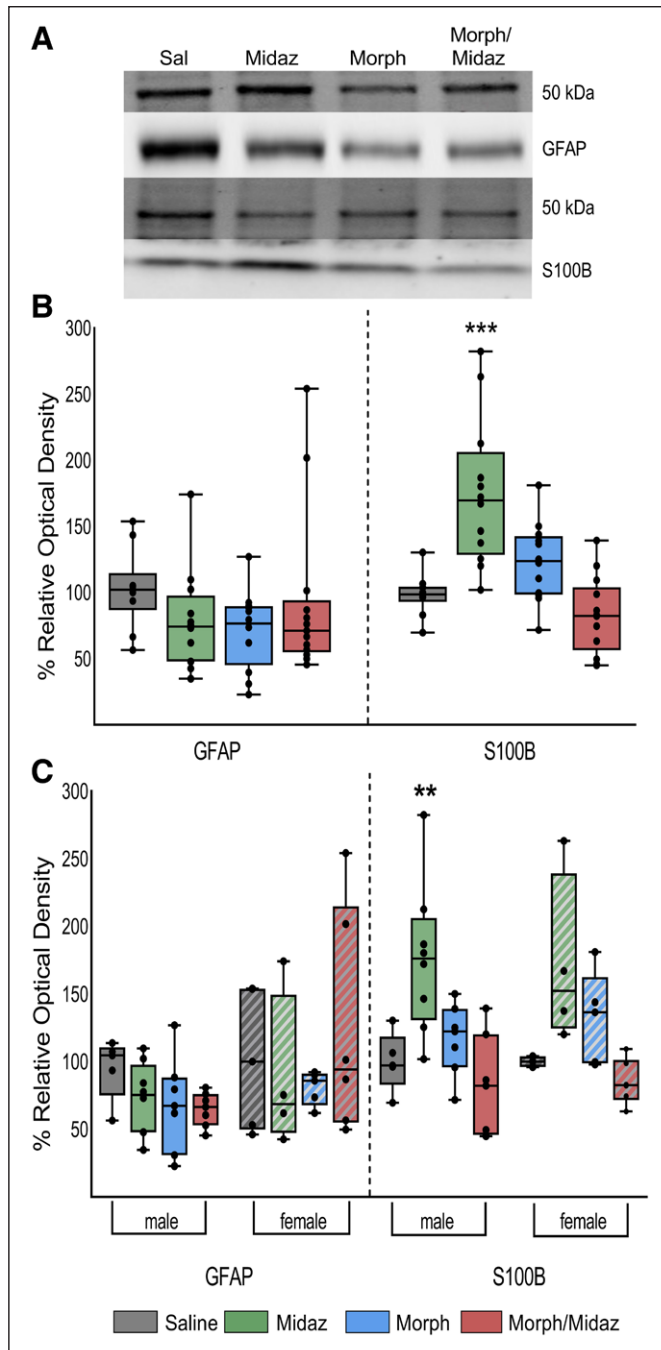


Figure 3. PICU-typical sedation and analgesia alters cerebral levels of astrocytic markers. Representative western blot and *box whisker* plots for glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein B (S100B) (A). The 50kDa total protein band was used as a loading and transfer control. Each treatment (midazolam [Midaz], morphine [Morph], morphine + midazolam [Morph/Midaz]) is compared with saline (Sal), which is set at a mean of 100. Combined sex group comparisons are shown in (B), whereas separate sex comparisons are shown in (C). Using Sal as a reference: ** $p \leq 0.01$, *** $p \leq 0.001$.

(Midaz 74.3% [IQR, 47.8–97.8] vs Sal 101.0% [IQR, 65.0–135.0], $p = 0.12$; Morph 76.7% [IQR, 45.1–89.7] vs Sal 99.6% [IQR, 64.1–136.0], $p = 0.09$; Morph/Midaz 71.2% [IQR, 54.8–94.2] vs Sal 100% [IQR, 94.7–127.0], $p = 0.05$). There was wide variability in females, and decreased median GFAP was more pronounced in all male treatment groups and Morph-treated females (Midaz: male 75.4% [IQR, 47.8–97.8] vs female 68.7% [IQR, 47.4–149.0], $p =$

0.92; Morph: male 67.4% [IQR, 31.0–88.4] vs female 85.9% [IQR, 67.8–91.2], $p = 0.36$; Morph/Midaz: male 66.5% [IQR, 53.0–76.1] vs female 94.2% [IQR, 55.0–215.0], $p = 0.08$).

Interestingly, as shown in **Figure 4**, median cerebral SYN expression was increased in Morph versus Sal (136.0% [IQR, 104.0–158.0] vs 92.7% [IQR, 91.1–108.0], $p = 0.05$). This was greater for males than females (males 152.0% [IQR, 106.0–195.0], $p = 0.03$; females 113.0% [IQR, 74.1–143.0], $p = 0.66$). Results also indicated that compared with Sal, treatment with Midaz and Morph/Midaz numerically increased SYN by 26% and 11%, respectively, without a male-female difference (Midaz 126.0% [IQR, 95.5–157.0] vs 94.8% [IQR, 82.1–121.0], $p = 0.07$; Morph/Midaz 111.0% [IQR, 92.3–163.0] vs 94.8% [IQR, 82.1–121.0], $p = 0.16$). Drebrin expression significantly increased only in Morph/Midaz versus Sal (119.0% [IQR, 110.0–127.0] vs 101.0% [IQR, 93.1–109.0], $p = 0.01$), although the upper quartile range for Morph (IQR, 88.0–135.0) is larger than Sal (IQR, 91.3–109.0). This Morph/Midaz effect was statistically significant in females but not males (female 123.0% [IQR, 116.0–127.0], $p = 0.04$; male 114.0% [IQR, 107.0–130.0], $p = 0.12$). Results also indicated that Midaz-treated females expressed significantly more drebrin than males (female 113.0% [IQR, 99.8–157.0] vs male 85.9% [IQR, 79.8–97.3], $p = 0.02$).

MBP results are depicted in **Figure 5** and showed significantly increased 17–18.5 kDa splicing variant after Midaz (17–18.5 kDa: 123.0% [IQR, 107.0–201.0] vs Sal 102.0% [IQR, 89.1–107.0], $p = 0.01$). This pattern was seen in both Midaz males and females (male 133.0% [IQR, 102.0–253.0], $p = 0.06$; female 123.0% [IQR, 117.0–154.0], $p = 0.07$). Midaz treatment also numerically increased 21.5 kDa splicing variant without a male-female difference (127.0% [IQR, 95.6–159.0] vs Sal 101.0% [IQR, 87.9–109.0], $p = 0.13$). There were no significant differences in the medians for 14 kDa MBP, although Midaz and Morph/Midaz had larger 75% upper quartile ranges (Midaz [109.0–176.0], Morph/Midaz [107.0–162.0]). Midaz males expressed numerically more 14 kDa MBP than females (male 132.0% [IQR, 99.3–190.0]; female 110.0% [IQR, 72.3–154.0]). Although there were no combined sex differences in MBP splicing variants after Morph treatment, expression was lower in males compared with females (14 kDa male 70.2% [IQR, 66.8–101.0] vs female 107.0% [IQR, 95.8–142.0], $p = 0.04$; 17–18.5 kDa male 82.6% [IQR, 66.8–101.0] vs female 101.0% [IQR, 90.5–142.0], $p = 0.09$; and 21 kDa male 74.6% [IQR, 54.9–111.0] vs female 108.0% [IQR, 84.4–161.0], $p = 0.07$).

As depicted in **Figure 6**, Midaz treatment numerically increased median microglial Iba1 compared with Sal (119.0% [IQR, 95.6–159.0] vs 98.6% [IQR, 95–107.0], $p = 0.12$), and Iba1 was numerically increased in Morph/Midaz (114.0% [IQR, 91.5–144.0] vs 100.0% [IQR, 95.6–104.0], $p = 0.21$). There was no Iba1 difference between Morph and Sal (98.2% [IQR, 83.8–123.0] vs 98.6% [IQR, 95.0–107.0], $p = 0.74$). Although there was more variability in females than in males, there were no significant differences between sexes.

DISCUSSION

The present results indicate that despite the low 5- to 9-mg/kg midazolam and 2.5- to 4-mg/kg morphine dose used in this PNDs 18–24 model, there are drug effects on established markers of brain

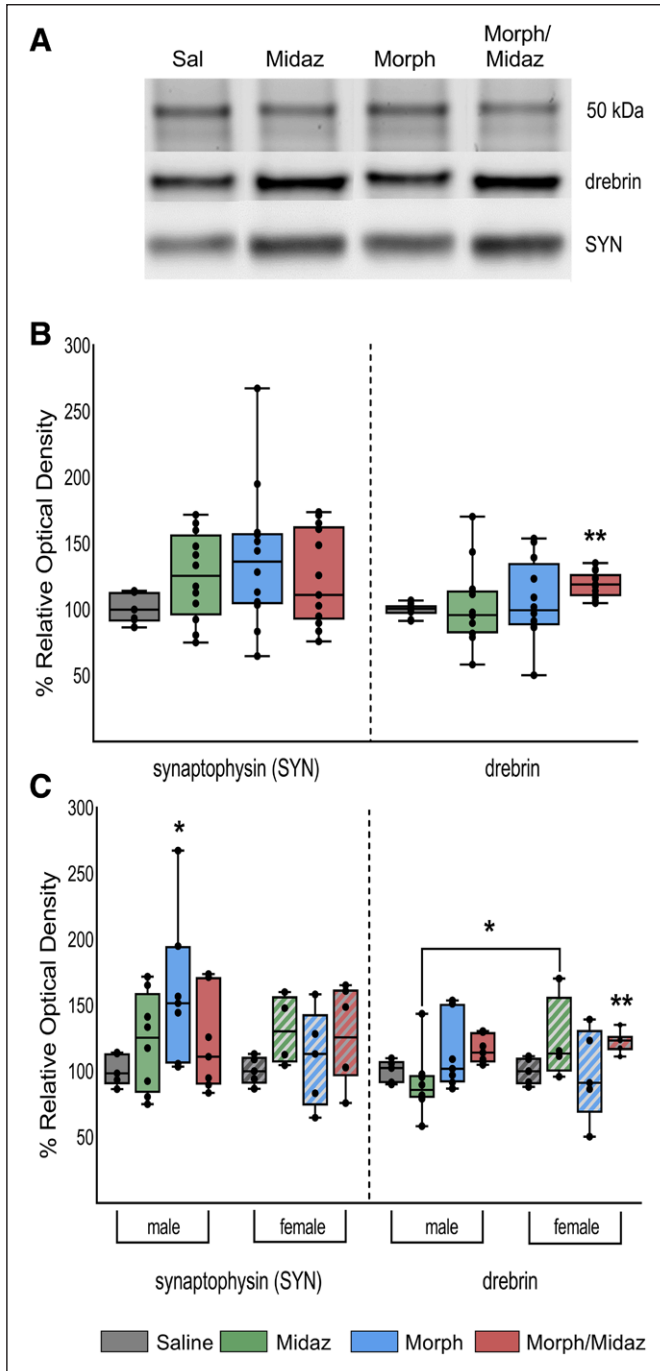


Figure 4. PICU-typical sedation and analgesia alters cerebral levels of synaptic markers. Representative western blot and *box whisker* plots for synaptophysin (SYN) and drebrin (A). The 50 kDa total protein band was used as a loading and transfer control. Each treatment (midazolam [Midaz], morphine [Morph], morphine + midazolam [Morph/Midaz]) is compared with saline (Sal), which is set at a mean of 100. Combined sex group comparisons are shown in (B), whereas separate sex comparisons are shown in (C). *Linking brackets* indicate statistically significant between sex differences. Using Sal as a reference: * $p < 0.05$, ** $p \leq 0.01$.

development and plasticity. More than 20% increase in the median presynaptic marker SYN was observed in both Morph and Midaz groups, and the SYN increase was higher in Morph males than in Morph females. There was more variability in the levels of the post-synaptic dendritic marker drebrin in Morph and Midaz-treated animals that disappeared with combined Morph/Midaz in males

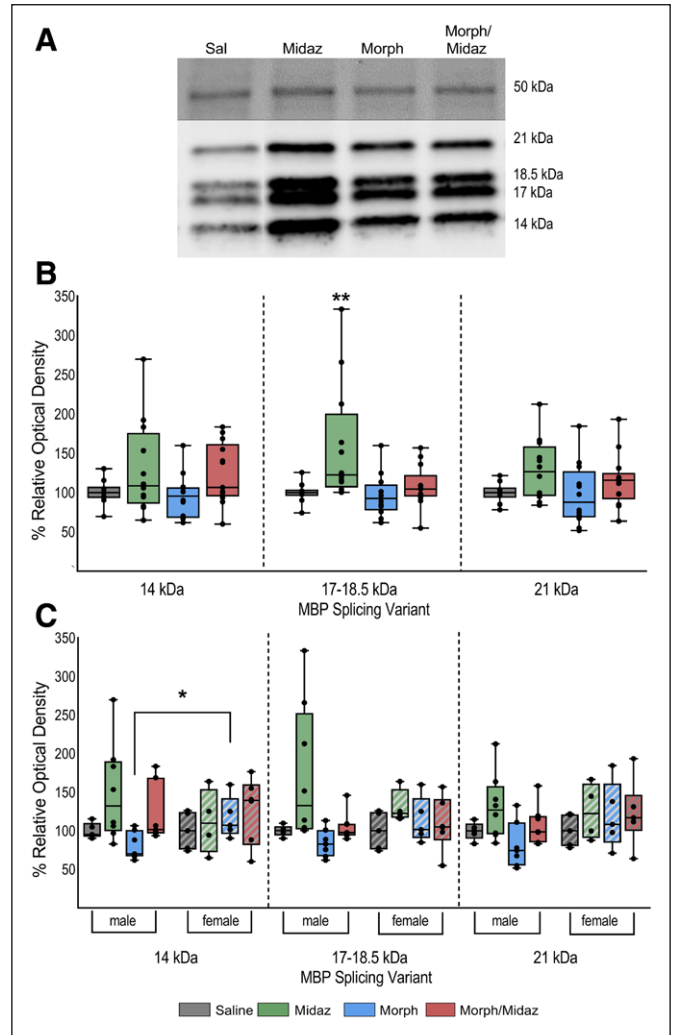


Figure 5. Midazolam exposure alters cerebral levels of myelin basic protein (MBP). Representative western blot and *box whisker* plots for developmentally regulated MBP splicing isoforms: 14, 17, 18.5, and 21 kDa (A). The 50 kDa total protein band was used as a loading and transfer control. Each treatment (morphine + midazolam [Morph/Midaz], morphine [Morph], midazolam [Midaz]) is compared with saline (Sal) control, which is set at a mean of 100. Combined sex group comparisons are shown in (B), whereas separate sex comparisons are shown in (C). *Linking brackets* indicate statistically significant between sex differences. Using Sal as a reference: * $p < 0.05$, ** $p \leq 0.01$.

and females. There was a significant drebrin increase in females and combined sex analysis. These results are in line with synaptic up-regulation and dendritogenesis reported by Xu et al (3) and Briner et al (17) and De Roo et al (18) using single-drug Midaz at a 10-25 mg/kg dose during PND 15-22, although Xu et al (3) reported decreased synapsin-1. Lack of uniform single-drug effect in our drebrin results is likely explained by the comparatively low dosing in outbred animals, but interestingly, there appears to be an interactive effect on the postsynapse even when low-dose opioid and benzodiazepine were combined in the Morph/Midaz group.

Benzodiazepines are suspect in sedative neurotoxicity, and they are associated with both synaptic dysregulation and delirium in a dose-dependent manner; however, opioids also modulate synapses (35–37). In our model, both presynaptic and postsynaptic markers are affected by Morph, alone or in combined Morph/Midaz. This

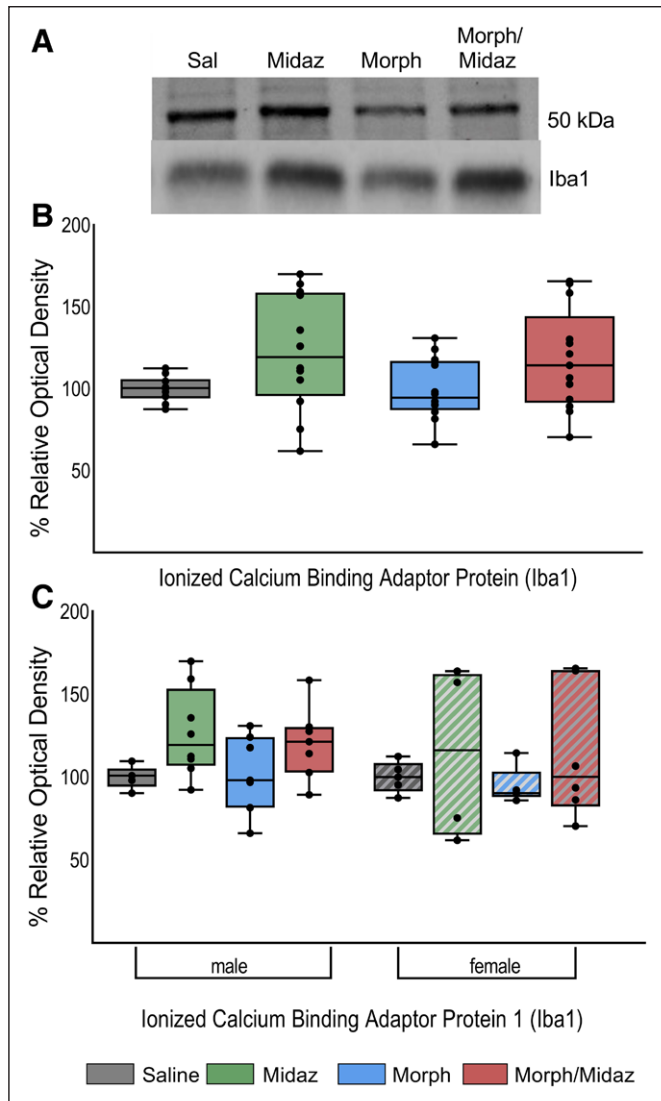


Figure 6. Midazolam and morphine + midazolam (Morph/Midaz) exposure and cerebral levels of ionized calcium-binding adaptor protein (Iba1). Representative western blot and *box whisker* plots for Iba1. The 50 kDa total protein band was used as a loading and transfer control. Each treatment (Morph/Midaz, morphine [Morph], midazolam [Midaz]) is compared with saline (Sal), which is set at a mean of 100. Combined sex group comparisons are shown in (B), whereas separate sex comparisons are shown in (C). Sal versus Midaz, Morph, and Morph/Midaz: not significant.

is similar to previously observed increases in striatal and cortical presynaptic and postsynaptic markers after morphine administration in mature rodents (38, 39). Morph administration blocks endogenous GABA-related neuronal inhibition, with likely interplay of benzodiazepines and opioids in PICU-typical sedation, the effects of which on the critically ill developing brain are unknown (40–42). Under normal conditions, dendritic synaptogenesis is experiential, and drug-promoted synapses may be disorganized, abnormally interconnected, or preferentially formed in brain regions related to reward and emotion, contributing to neurologic dysfunction such as delirium or posttraumatic stress disorder.

Relevant literature has shed light on neuronal viability and dendritic synaptogenesis; however, our results also point to glial effects that warrant directed study. In support of this, when serum

biomarkers are correlated with poor neurologic outcome in PICU patients, not only is neuron-specific enolase a potential predictor, but so are S100B, GFAP, monocyte chemoattractant protein 1, and oligodendrocytic MBP (43, 44). In particular, astrocytes regulate synaptic plasticity and activity and provide ionic and neurotransmitter homeostasis. Astrocytes also supply metabolic substrate, balance extracellular fluid, and modulate cerebral blood flow (45). In our model, median cerebral GFAP expression was reduced by more than 20% in Morph/Midaz, Morph, and Midaz groups. Reduced GFAP expression may reflect stunted astrocyte outgrowth or activity and deserves further study to elucidate mechanism. Irrespective of mechanism, given their homeostatic role, astrocyte abatement by sedatives in the face of critical illness brain stress could contribute to neurologic sequelae (46). In contrast to GFAP, the mature astrocyte marker S100B is significantly and numerically increased in Midaz and Morph groups, respectively. Because S100B is expressed by mature astrocytes, preserved or increased brain S100B argues against reduced astrocyte population to explain reduced GFAP and may indicate drug-altered astrocyte maturation (34, 47). Importantly, S100B is also secreted by astrocytes, which, at physiologic levels, regulates neurite outgrowth and synaptic plasticity, and inhibits astrocyte and microglial activation and inflammation (48). In contrast, at high levels observed after brain injury, S100B activates inflammatory microglia and astrocytes, inhibits oligodendrocyte maturation, and alters synaptic protein expression. Notably, elevated serum S100B has been correlated with delirium and postdelirium encephalopathy, and the largest effect in our study was with benzodiazepine treatment (49). S100B has demonstrated utility as a clinical biomarker for a variety of brain injuries, and given its role in synaptic plasticity and myelination in the developing brain, potential modulation by PICU-typical sedation is an important result for directed study.

The numeric increase in microglial Iba1 in Midaz and Morph/Midaz with no change in Morph was unexpected in our model, given known opioid activation of the microglial toll-like receptor 4 pathway, and reported impaired intellectual quotient testing in meningococcal shock survivors who received opioids but not benzodiazepine (50, 51). This result may be related to low Morph dose and/or lack of concomitant injury or inflammation and may reflect increased trophic microglial chaperoning of accelerated synapse formation induced by benzodiazepine GABA agonism rather than reactive inflammatory microglia (52, 53). Although Midaz increased both Iba1 and S100B, Morph/Midaz increased Iba1 without S100B increase. This may reflect drug interaction or dose-dependent S100B and Iba1 effects below an inflammatory threshold. Future measurement and brain localization of inflammatory mediators such as cytokines should shed light on the mechanism of increased Iba1 after GABA agonism.

Finally, although both endogenous opioid and benzodiazepine are involved in oligodendrocyte maturation and axonal myelination, only exogenous GABA agonism with midazolam significantly increased MBP expression in our model (54). Given previous observations of accelerated rat brain myelination after perinatal exposure to low doses of buprenorphine and methadone, we had anticipated morphine effect (55, 56). Our present results may reflect age-dependent differences in myelin regulation

or insufficient morphine dose. It should be noted that S100B is also expressed by immature oligodendrocytes, and the S100B increase in the Midaz group may not be solely astrocytic given increased MBP (57). Taken together, it is evident that although neuronal health is an important target for future investigation, glial cells are a reservoir for neurologic resilience and a potential therapeutic target during critical illness that may be influenced by PICU-typical sedation.

It is striking that although Morph animals were minimally sedated compared with Morph/Midaz, weight gain was limited in both, and to a greater degree in Morph. Nutritional access was identical across groups, suggesting opioid behavioral effects on appetite or resource acquisition. Our model is similar to reports of oral motor and other behavioral stimulant effects after morphine administration in rodents, cats, and primates, as well as disrupted typical rodent diurnal patterns and reduced nighttime eating with associated weight loss (58–60). Our observations suggest an unknown duration of opioid effects on behavior and possibly sleep/wake patterns that may impact neurodevelopment or signal neuropathology. Malnutrition can retard brain development, particularly myelination, and Morph males gained the least weight and numerically expressed the least MBP; however, MBP expression was not altered in female Morph or male and female Morph/Midaz animals that also gained less weight than Sal or Midaz (61, 62). Our study lacks an underweight control group to clarify the contribution of nutrition. However, achieving weight loss in preweaning rats before PND 18 necessitates premature weaning or maternal separation which have confounding neurodevelopmental consequences (63). This might be addressed in future studies by shifting the first experiment day by several days to postweaning; however, rodents are coprophagic, and specialized measures may be necessary.

Limitations of our study include intermittent, comparatively low dosing. This was necessary to establish a combined sedative and analgesic model, and our findings may underestimate dose-dependent effects. Continuous infusions with implantable pumps were considered but would not achieve a PICU-typical depth of sedation and require a confounding anesthetic exposure. Low-range dosing is not only required to establish dose-dependent responses but also is necessary for developing combination models of sedation and critical illness in which animal viability is a concern. Noninvasive oxyHb% ruled out hypoxemia, a critical parameter for brain injury. However, the need for supplemental oxygen in our model suggests hypoventilation, and lack of blood gas measurement of carbon dioxide is a limitation. Marked hypercarbic respiratory acidosis is routinely tolerated as a lung-protective strategy in contemporary critical care, and acidosis may affect cellular physiology in addition to cerebral blood flow in the developing brain, particularly if supportive glial function is altered by illness or medication. Because carbon dioxide is a robust regulator of vascular tone and cerebral blood flow, future and similar studies should include blood gas measurement, or if feasible, assessment of cerebral blood flow with near-infrared spectroscopy scaled for rodents. Biologic variability in vivo is expected in outbred rats undergoing dynamic neurodevelopment, and our sample size may have, therefore, been too small to statistically detect differences, particularly at this low range of drug dosing.

We analyzed groups by sex, and fewer females per litter resulted in fewer females per some group analysis, limiting interpretation between sex results. Nonetheless, what differences we observed highlight the importance of including both sexes in these experiments, even in prepubertal pups. It would also be useful to include both younger than PND 15 and older than PND 35 groups to establish a timeline of age-dependent effects. Another limitation is inclusion of a single acute timepoint in this study because there are likely subacute (adolescent) and chronic (adult) changes. Finally, we used total hemispheric homogenate, which likely obscured anatomically localized effects. Complementary tissue analysis for regional effects will be important in follow-up studies, particularly in brain regions corresponding to observed clinical outcomes. Nonetheless, the present model suggests important changes in synaptic and glial protein expression that certainly warrant further investigation given increasing evidence that survivors of childhood critical illness incur neurologic morbidity and mortality.

Advances in modern critical care medicine have shifted attention from simple survival to meaningful recovery and health-related quality of life (HRQL). PICU-associated neurologic morbidity is prevalent, and survivors suffer a variety of neurologic sequelae, from delirium and reduced Pediatric Cerebral Performance Category scores, to reduced HRQL, a spectrum of impaired learning and memory, posttraumatic stress, and behavioral difficulties (25–29, 64–69). Notably, not all neurologic consequences manifest immediately or as overt deficits, even in animal models (13). In addition to acute sequelae, critical illness brain stress and therapies, including sedation and analgesia, may create psychological, sociability, or executive functioning vulnerabilities that accumulate over time or manifest remotely under stress or stimulus. Subtlety and latency may explain heretofore under-recognized neurotoxicities, and survivors may also be at risk as they age. Critical illness inherently involves a complex variety of factors that impact the developing brain, and there are gaps in our knowledge with regard to the most routine of contemporary therapies. Understanding the contribution of sedatives and analgesics, for better or worse, is a necessary step in developing patient-based and disease-based neuroprotective strategies for optimizing outcomes for the uniquely vulnerable PICU population. We have been lacking preclinical models to that end. Considering the study limitations discussed, future investigation should target dose-dependent effects on both neuronal and glial cell populations, interactivity with concomitant critical illness brain stress and injury, and age-dependent effects in infants, children, and adolescents.

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