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Early developmental exposure to repetitive long duration of midazolam sedation causes behavioral and synaptic alterations in a rodent model of neurodevelopment

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Abstract

There is a large body of preclinical literature suggesting that exposure to general anesthetic agents during early life may have harmful effects on brain development. Patients in intensive care settings are often treated for prolonged periods with sedative medications, many of which have mechanisms of action that are similar to general anesthetics. Using *in vivo* studies of the mouse hippocampus and an *in vitro* rat cortical neuron model we asked whether there is evidence that repeated, long duration exposure to midazolam, a commonly used sedative in pediatric intensive care practice, has the potential to cause lasting harm to the developing brain. We found that mice that underwent midazolam sedation in early postnatal life exhibited deficits in the performance on Y maze and fear conditioning testing at young adult ages. Labeling with a nucleoside analog revealed a reduction in the rate of adult neurogenesis in the hippocampal dentate gyrus, a brain region that has been shown to be vulnerable to developmental anesthetic neurotoxicity. Additionally, using immunohistochemistry for synaptic markers we found that the number of pre-synaptic terminals in the dentate gyrus was reduced, while the number of excitatory post-synaptic terminals was increased. These findings were replicated in a midazolam sedation exposure model in neurons in culture. We conclude that repeated, long duration exposure to midazolam during early development has the potential to result in persistent alterations in the structure and function of the brain.

Keywords

Anesthesia; Neurotoxicity; Synapse; Learning; Neurodevelopment

Introduction

Evidence from studies conducted in animal models, including rodent^{1–9} and non-human primate e.g.^{10–13}, suggests the possibility that early developmental exposure to general anesthesia (GA) has harmful consequences for brain development that lead to subsequent cognitive dysfunction. Furthermore, several large retrospective epidemiologic studies show an association between early childhood exposure to GA in the setting of surgery and evidence of impaired cognitive function, including increased incidence of learning disorders and worsened performance on school assessments^{14–17}. While not all studies replicate this finding^{18–20}, perhaps due to variations in both exposures and outcomes which have been studied, the concern that these medications might cause neurotoxicity has led the U.S. Food and Drug Administration (FDA) to issue a safety communication²¹. Although nearly all the evidence in human studies and animal models relates to GA as practiced in operating room (OR) settings, the FDA also warns of risks associated with medications described as “sedation drugs” used during surgery and other procedures. This leaves open an important question with potentially substantial public health ramifications: Does sedation for infants and young children as practiced in intensive care unit (ICU) settings have the potential to cause harm to the developing brain?

Although there are some features shared between GA for surgery and sedation in the ICU, there are key differences that make it difficult to predict whether sedation would be expected to have similar neurodevelopmental outcomes. The commonly used drug classes include benzodiazepines, opiates, and increasingly α 2-adrenergic agonists, all of which are used as adjuncts rather than principal agents in GA. In rodent models, several studies found that phenotypic alterations in vitro and in vivo, such as cell death and loss of growth cone sensing resulting from midazolam exposure alone^{3, 22–25}. However, another in vitro study found that midazolam failed to alter dendritic development in a paradigm where propofol exposure led to reduced arborization²⁶. The duration of exposure may be the most critical difference between ICU sedation and OR GA, as ICU sedation can continue for days or even weeks. A recent study conducted in a pediatric cardiac intensive care unit (PCICU) found an association between the use of benzodiazepines and the development of delirium²⁷. Recently published clinical trials suggest that short exposures to GA in the OR ranging from 30 minutes and up to 2 hours in healthy children are not associated with cognitive deficits^{28–30}. This is supported by retrospective studies, in which long duration and/or multiple exposures to GA are required for a significant association with subsequent cognitive dysfunction.^{14, 16, 17, 31, 32}. The mechanisms of sedative toxicity are likely to overlap with those of anesthetic neurotoxicity, but currently there is no clear consensus of what those mechanisms might be, although some candidates include cell death, disruptions in axon or dendritic growth, and alterations in synapse structure and function³³. Because of the potential similarities between ICU sedation and OR GA, and the absence of any clear and comprehensive literature on this topic, there is a compelling rationale to study the effects of ICU-type sedation on the developing brain.

To investigate the potential effects of ICU sedation exposure on neurodevelopment, we have established both an *in vivo* mouse model of long duration exposure to midazolam, a commonly used pediatric sedative, and a parallel *in vitro* model using primary cultured

neurons. We used the *in vivo* model to ask whether early developmental exposure to chronic, repeated, long duration midazolam titrated to sedative doses results in lasting neurobehavioral changes that indicate a disruption of brain function. We further employed both the *in vivo* and *in vitro* models to test for structural consequences of our midazolam exposure model, including changes in neurogenesis and synapse formation.

Materials and Methods

Animal treatment and handling

Animals—C57BL/6 mice were housed in a temperature- and humidity-controlled room with a 12:12 hour light: dark cycle, and provided with ad libitum access to water and food. Both sexes were equally represented in all experiments. No animals were excluded. All study protocols involving mice were approved by the Animal Care and Use Committee at the Johns Hopkins University and conducted in accordance with the NIH guidelines for care and use of animals.

Midazolam treatment and physiologic monitoring of sentinel animals—The exposures were conducted during daytime hours (07:00–19:00) and repeated for 5 days, from P18 to P22. The experimental room was temperature-controlled, so that this room had the same temperature and humidity as compared to the animal room. We performed behavioral tests in 6 male (weight 7.43 ± 0.50 g) and 6 female (weight 7.46 ± 0.27 g) mice per group, and harvested brains for immunohistochemistry in three male and three female animals per group (Figure 1A). During the experiment, mice received periodic intraperitoneal injections according to protocol, and were kept on a warming pad maintained at approximately 38°C for the duration of the experiment, as well as the recovery time. Exposures were performed at P18. This age range was selected because it roughly equates to early childhood in terms of brain development³⁴, and also because mice were robust enough to show minimal physiologic and nutritional perturbations in our exposure model. Two different doses (10mg/kg and 20mg/kg) were chosen based on pilot studies to determine what dosing was required to achieve moderate and deep sedation based on the rating scale (Table 2). The duration of exposure, which was selected based on pilot experiments showing what duration could be used without causing overt signs of nutritional or physiologic perturbation (as assessed by changes in weight, heart rate, oxygen saturation and temperature) likely corresponds to a longer exposure in humans, provided the differences in developmental timelines. Littermates were randomly assigned to 3 groups: Group 1 (naïve control): Mice were separated from the dams for 12 hours but did not receive injections; Group 2 (midazolam 10mg/kg): Mice were treated with 10 mg/kg midazolam (Hospira Inc., Lake Forest, Illinois, USA) per hour; Group 3 (midazolam 20mg/kg): Mice were treated with 20 mg/kg midazolam every 2 hours. The injection was performed at a clean bench in a sterile manner. Midazolam was mixed with sterilized saline to adjust it to a uniform volume of 100µl /10 g b.w./mouse. The drug was prepared before each injection and kept at the same temperature as the mouse body temperature (38°C) until administration. After completion of the experiment, mice recovered on the heating pad (Kent Scientific, Torrington, CT, USA) and were returned to their home cages upon regaining righting reflex. Mice were continuously monitored and skin temperature, heart rate, and O₂ saturation were measured

during the 12h midazolam treatment (PhysioSuite, Kent Scientific) (Table 1). Measurements for the control mice were not obtained due to difficulty securing un-sedated pups in the PhysioSuite unit. A sedation score for each mouse was measured every 15 minutes from the first injection until recovery following sedation.

Measuring sedation scores—Each anesthetic score was measured using the modified grading system described by Kawai³⁵ and Kirihara³⁶. Measurement was based on 4 reflex scales which are described in detail in Table 2. The total sedation score was graded from 0 to 9 (Scoring of control animals maintained at 0). The mice in group 2 (10mg/kg) scored between 2 and 3, which was considered to indicate a moderate sedative level in mice. A total score of the group 3 (20mg/kg) was 5–7, which was considered to indicate a deep sedative level in mice (Table 3).

Behavior tests

Mice were housed in standard conditions until P60 and then handled by the experimenter for at least 2 min per day for 3 days before the start of the behavioral experiments. Behavior was tested at P60, at which time mice are reproductively mature and brain development roughly correlates with early adulthood³⁴. All behavioral tests were performed in the animal behavior core at Johns Hopkins School of Medicine during the light phase of the cycle between 08:00 and 18:00. Experimenters were blind to condition when behavioral tests were carried out and analyzed. The numbers of mice per condition are indicated in the figure legends. Two commonly used behavior tests for measuring spatial memory function that is based in the hippocampus, the Y-maze and the fear-conditioning test, were performed^{4,37}.

The Y-maze test was performed as previously described⁴. In short, in the first phase, a mouse was released from the start arm (contained no visual cue) and allowed to explore only 1 out of 2 possible choice arms (each choice arm contained an overt visual cue) for 15min (Fig 2A). In the recognition testing phase, which occurred 24hrs later, the animal was released from the start arm and had access to both choice arms for 5 min. The testing phase was video recorded and percentage of exploration time in each choice arm was measured by an observer blind to condition.

The fear-conditioning test was performed according to a commonly used paradigm (Fig 2D)³⁷. On the day of the test, the mice acclimated to the room for 1 h prior to the assay. Each mouse was then placed into the tone-shock chamber (Coulbourn Instruments, Holliston, MA, USA, Fig 2C). The training paradigm used was 2min of acclimation in the chamber prior to a 30 s tone which was followed by a 0.5 mA shock. Then there was a 2min consolidation period, followed by a second tone-shock pairing, and this process was repeated for a third time. The criteria for the exclusion of a mouse from testing was immobility for >30% of the pre-shock habituation/acclimation phase, in order to avoid misinterpreting low habitual mobility as freezing. However, all the animals showed normal mobility during the experiment. Freezing was defined as the complete absence of motion, including motion of the vibrissae, for a minimum of 0.5s. Twenty-four hours after training, the mice were reintroduced into the same chamber for a period of 5 min to quantify contextual fear conditioning (both the people conducting the experiment and the people

scoring the experiment were blinded to the treatment condition). Approximately 1 h following the contextual assay, mice were placed into a novel apparatus which contained a 5% acetic acid scent, where a 30s tone was played for a period of 1 min. In both situations, the outcome measure was the amount of time spent freezing in response to the context or cue. The testing phase was video recorded and percentage of freezing time was measured by FreezeScan software (FreezeScan, Clever Sys Inc., Reston, VA, USA). This software assesses freezing by measuring changes in the intensity of each pixel between successive frames of the video file.

Neuronal Cultures—Primary neuron cultures were obtained from BrainBits, LLC (Springfield, IL, USA). Dissociated neurons were cultured from neocortex dissected from E18 Sprague Dawley rat embryos according to company protocols. Neurons were plated on 12 mm glass coverslips at 16,000 cells/cm². Cells were cultured in NbActiv4 medium (BrainBits, Springfield, IL, USA) under standard conditions and half media changes were conducted three times per week. Pilot experiments showed over 95% of cells from these cultures are immunopositive for β -tubulin, suggesting a high degree of purity. Experiments were performed on neurons between 7 and 10 DIV (days *in vitro*), and all experiments used coverslips from a minimum of 6 separate cultures.

Midazolam treatment for the neuronal cultures—The treatment was conducted by adding a range of midazolam doses (50nM, 100 nM, 150 nM) (Hospira Inc., Lake Forest, Illinois, USA) into experiment wells, and incubating the wells at 37°C with 5% CO₂ in the incubator (Panasonic Healthcare, Wood Dale, IL, USA) at 9:00 am on 7DIV. Half of the media volume was replaced on 8DIV and on 9DIV, and a full dose of the drug was added to maintain the dissolved concentration. The experiment was ended by fixing the cells using 4% paraformaldehyde in PBS at 9:00 am on 10DIV (Figure 1B).

Immunohistochemistry (IHC).

Fluorescent immunocytochemistry for *in vivo* experiments was performed in accordance to the previous protocol³⁸. BrdU was administered via intraperitoneal injections once per day at a 50mg/kg dose for animals randomly selected from three groups (n=4 each group) during P60 to P62. At P63, these mice were transcardially perfused with an initial 40 ml of ice-cold 0.1M PBS (pH 7.3). Brains were removed and then fixed in 4% paraformaldehyde in PBS at 4°C overnight, followed by cryoprotection in a 30% sucrose in PBS at 4°C until the tissue sank to bottom of the vials. The brains containing the dentate gyrus of hippocampus were coronally sectioned in 50 μ m thickness using a microtome (Leica SM2010 R sliding microtome, Buffalo Grove, IL, USA). The sections of each brain were collected in a rotating order and stored at -20°C in anti-freeze media (30% ethylene glycol and 15% sucrose in PBS) in a 96-well cell culture dish. 4–5 sections were picked evenly from the anterior, middle and posterior part of the hippocampus for the future staining. The sections were first washed in PBS (3 $\times$ 5 min). In BrdU staining, sections were pretreated with 2N HCl to denature DNA (37°C; 45min), and with 2 $\times$ 15-min borate buffer treatments (pH 8.5) to neutralize the HCl. Following blocking for 60 min in 10% normal donkey serum (NGS) and 0.1% triton X-100 in PBS, sections were incubated in primary antibodies at 4°C for 72h. Primary antibodies used in this study were: rat anti-BrdU (1:600, Abcam, Cambridge, MA,

USA), mouse- anti-NeuN (1:200, EMD Millipore, Burlington, MA, USA), rabbit-anti-synapsin-1 (1:200, EMD Millipore, Burlington, MA, USA), chicken-anti-homer-1 (1:400 , Synaptic Systems, Goettingen, Germany), and mouse-anti MAP-2 (1:200,EMD Millipore, Burlington, MA, USA). After 3×5min washes in PBS, sections were incubated with the following secondary antibodies: a combination of Alexa Fluor 488- or Alexa Fluor 594-labeled anti-rat, anti-chicken, anti-rabbit or anti-mouse secondary antibodies (1:250) and 4', 6'-diaminodino-2-phenylindole (DAPI, 1:5000) at room temperature for 2h. After 3×5min PBS washes, sections were mounted onto slides using 2.5% PVA/DABCO Mounting Media, air-dried, and cover-slipped.

Fluorescent immunocytochemistry for *in vitro* experiments were conducted as previously described²². Neurons on coverslips were briefly fixed with 4% paraformaldehyde at room temperature for 10 minutes, then permeabilized and blocked for 1hr at room temperature in 5% donkey serum with 0.1% Triton X- 100. Neurons were incubated overnight at 4°C using the following antibodies: Rabbit-anti-synapsin-1 (1:200, EMD Millipore, Burlington, MA, USA), Chicken-anti-homer-1 (1:400, Synaptic Systems, Goettingen, Germany), and Chicken-anti-MAP-2 (1:200, Abcam, Cambridge, MA, USA). All the antibodies were diluted in phosphate-buffered saline solution containing 0.1% Triton X-100. After rinsing, neurons were incubated for 2hrs with a fluorescent secondary antibody and 4',6'-diaminodino-2- phenylindole (DAPI, 1:5000) at room temperature for 2h at the manufacturer's recommended concentration (Jackson Immuno Research Labs, West Grove, PA, USA). Subsequently, neurons were mounted on coverslips using 2.5% PVA/DABCO Mounting Media.

Imaging and analyses

For the *in vivo* study, the immunostained sections were observed and imaged on a Leica SP8 confocal microscope (Leica, Wetzlar, Germany). The images for the dentate gyrus synaptic marker analysis were taken using a 63× 1.0 N.A. objective with an additional 5x magnification lens in line. All single- and double- immunolabeled signals/ cells in this area were quantitatively analyzed using ImageJ program (NIH, Bethesda, USA). The sections were processed in parallel and images were acquired using the identical settings in order to allow for comparable measurements. Fluorescence intensity was measured in ImageJ and the value was normalized to background signal in the same image. These data were then normalized to the area of the dentate gyrus granule layer as defined by DAPI staining. Three sections were picked randomly from each animal, representing the rostral, middle and caudal part of the hippocampal dentate gyrus. For the synaptic marker images, 3 images were taken for each slice and at random locations in the dentate gyrus, as defined by DAPI staining.

For *in vitro* study, five neurons from fields that were evenly distributed throughout the coverslip to represent all four quadrants and the center were randomly selected for analysis. Images were taken using a 63× 1.0 N.A. objective with an additional 5x magnification lens in line. Dendrites were identified by MAP-2 immunolabeling, the region of the dendrite which was analyzed was a segment 20–30µm from the nuclear rim according to DAPI staining.

All experimental analysis was carried out by personnel blind to condition. J.X. and R.P.M. contributed to behavior tests, J.K., J.J.L. and S.J. contributed to IHC, J.X. contributed to imaging, E.B. and S.S. contributed to image quantification, J.X. contributed to data analysis.

Statistical analysis

Results were expressed as mean $\pm$ SEM. Statistical analysis was conducted using Prism 6.0 Software (GraphPad Inc.). Immunohistochemistry data were analyzed using one-way ANOVAs with multiple comparisons, Y-maze data were analyzed using t-tests, and fear conditioning data were analyzed using a one-way ANOVA with Dunnett's multiple comparisons post-hoc test. The sample sizes were indicated in figure legends. The data that was examined with parametric test was determined to be normally distributed, and the criteria for statistical significance was set a priori at $p < 0.05$.

RESULTS

Midazolam sedation impairs spatial learning and memory.

To determine whether exposure to midazolam leads to long term deficits in cognitive function we employed Y-maze and fear conditioning paradigms, both of which have previously been used to identify effects on learning mediated by GA exposure during development^{4, 37, 39}.

In the Y-maze test (Fig 2A), control animals spend more time exploring the novel arm than the original arm ($p < 0.05$), as expected. In contrast, animals in 10mg/kg midazolam sedation group do not exhibit a preference for exploration of the novel arm as compared to the original arm. Similarly, the 20mg/kg midazolam group did not show differences in exploration time between the original and novel arms (Fig 2B). This data suggests a deficit in learning and memory function in the mice exposed to early developmental midazolam sedation at both the low and high doses.

To confirm our findings in the Y-maze test, we conducted contextual and cued fear conditioning (Fig 2C). This learning and memory testing paradigm differs from the Y-maze in several ways, including a lack of dependence on locomotor or visual function. In the contextual test, when compared to the controls, both low ($p < 0.05$) and high ($p < 0.001$) dose groups of the midazolam sedated mice showed reduced freezing time percentages (Fig 2D). In the cued test, when compared to controls, both the 10mg/kg midazolam sedation group ($p < 0.05$) and the 20mg/kg midazolam sedation group ($p < 0.001$) showed reduced freezing time (Fig 2E). Taken together, these data constitute strong evidence that our midazolam sedation paradigm results in a lasting deficit in learning and memory for sedated mice as compared to naïve controls.

Early postnatal midazolam sedation exposure has lasting effects on neurogenesis

Deficits in spatial learning and memory in rodents can be attributable to a wide variety of lesions, and the challenge to investigating a deficit due to an early postnatal toxic exposure is identifying an effect that persists into adulthood. Reduced performance in both the y-maze and fear conditioning paradigms typically results from impaired hippocampal function. The

dentate gyrus of the adult hippocampus is the site of ongoing neurogenesis throughout life. In rodents this neurogenesis is required for spatial learning, and it has been proposed as a possible target for GA toxicity⁴⁰. In order to investigate possible mechanisms of sedation toxicity, we first asked what the long-term effect of early sedation exposure is on dentate gyrus neurogenesis using 5-bromo-2'-deoxyuridine (BrdU), a synthetic thymine analog. Mice that had undergone early midazolam sedation from P18 to P22 and naïve control mice were injected with BrdU at P60 and assayed for neurogenesis at P63.

Representative confocal images of the dentate gyrus are shown with immunolabeling for BrdU (green) and the neuronal nuclear marker NeuN (red) at P63 (Fig 3A and 3B). The dentate gyrus granule layer was defined via DAPI staining. Total cell numbers and numbers of BrdU+/NeuN+ cells, which represent recently generated neurons and thus are a measure of ongoing neurogenesis, were quantified within the granule cell layer. Results were expressed as cells per unit area. Compared to the control group, there is a decrease in BrdU +/ NeuN+ positive cells number in both the 10mg/kg ($p<0.05$) and the 20mg/kg ($p<0.05$) midazolam sedation groups (Figure 3C). These data suggest a lasting decrease in neurogenesis that results from early life exposure to midazolam sedation.

Midazolam sedation results in lasting changes in dentate gyrus synapses

Normal synaptic function in the hippocampus is critical for spatial learning in rodents. The dentate gyrus of the hippocampus, within which we have already identified a defect in neurogenesis that is a result from our sedation model, is a late-developing brain region in which synaptogenesis is ongoing at a rapid pace during postnatal life due to formation of newborn neurons⁴¹. To evaluate the effects of our mouse model of sedation on the dentate gyrus, we conducted immunohistochemistry at P63 for Synapsin-1, a universal pre-synaptic marker⁴², and Homer-1, a marker of excitatory post-synaptic elements⁴³, on brain sections from sedated and control mice. Representative images for Synapsin-1 (green) and Homer-1 (red) *in vivo* studies are shown in Figure 4. Representative examples of colocalization of pre- and post- synaptic markers are indicated by the blue arrows (Fig 4A). As shown in Fig 4B, the number of colocalized pre and post-synaptic puncta are decreased in the midazolam groups compared to the control one ($p<0.01$). When we analyzed the pre-synaptic marker and post-synaptic marker separately, we found that for Synapsin-1, there is a decrease in puncta number between the midazolam groups and the control group ($p<0.05$ for 10ng/kg, $p<0.01$ for 20mg/kg), and the fluorescence intensity showed a similar effect relative to controls ($p<0.05$ for 10ng/kg, $p<0.01$ for 20mg/kg) (Fig 4C). In contrast, there is an increase in Homer-1 puncta number between the midazolam groups and the control one (Fig 4D), while the puncta intensity also showed a slightly but significant increase in the midazolam groups relative to the controls (Fig 4E). These findings represent a substantial, lasting change in the normal composition of synaptic elements in the dentate gyrus that may result from long-term sedation exposure to midazolam.

Synapse formation *in vitro* is disrupted following long-duration midazolam exposure

The process of synapse formation *in vivo* in the dentate gyrus occurs over a very long period of time and populations of synapses in all stages of development and maturity exist at P18. Thus, it is not possible to say from the experiments described above whether the midazolam

exposure affected synapses that had already formed, those which were in the process of forming, or those which had not even been generated yet. Neurons in dissociated culture develop along a fairly synchronous timeline in which synapses first begin to develop at 5 days in vitro (DIV), and pre- and post-synaptic elements meet and stable synapses are formed mostly between P7 and P10.

To better understand the effects of long duration midazolam exposure on synaptogenesis we conducted a 72-hour midazolam exposure on dissociated neurons in culture from 7DIV to 10DIV as a model for sedation exposure and subsequently conducted quantitative IHC to measure synaptic markers at 10DIV. Representative images of Synapsin-1 and Homer-1 immunolabeling are shown in Fig 5A with MAP-2 labeling to define dendrites. We measured the intensity of these two markers in punctate structures, representing synapses, as a function of dendritic length in segments adjacent to the cell body. These exposures were conducted across a range of doses including 50nM, 100nM, and 150nM⁴⁴ while the control group was treated with vehicle only. We found no significant changes between synapsin-1 puncta number between the low midazolam dose groups and the control one, while the 100nM group and 150nM group ($p<0.01$) showed a decrease (Fig 5B). When we compared the fluorescence intensity of synapsin-1, there is a significant decrease of the pre-synaptic marker intensity in the 100nM and 150nM groups compared to the control and to the 50nM midazolam group in which there were no changes in synapsin-1 intensity (Fig 5C). For Homer-1 puncta number, there was an increase in the 100nM midazolam and 150nM midazolam groups compared to the control, and 50nM midazolam groups (Fig 5D). Puncta intensity measurements showed the same effect for the 100nM midazolam group ($p<0.05$) and 150nM midazolam group ($p<0.01$) compared to the control, while 50nM midazolam did not significantly change in puncta intensity when compared to the control (Fig 5E). These findings closely mirror those derived from *in vivo* experiments, and suggest that the decrease in pre-synaptic profiles and increase in excitatory post-synaptic profiles occur during synaptogenesis, and that this defect is not repaired over time.

DISCUSSION

In this study we used a mouse model to address the question of whether early life exposure to sedative medications delivered over long periods of time, as occurs in ICU settings, can result in neurotoxic effects that are comparable to those which have been found for early life exposure to GA. We found that both moderate and deep sedation with midazolam, a commonly used pediatric ICU sedative medication, results in a lasting impairment of performance on behavioral tasks designed to test learning and memory. The Y- maze and fear conditioning tests employed here are complementary assays and the deficits in both these assays strongly suggest the possibility of a deficit in hippocampal function. The hippocampus is a late developing brain structure across mammalian species¹⁵, and thus it is a potential target for early life toxic exposures. Having established a behavioral phenotype of developmental sedation toxicity, we then moved to understand whether there were any lasting changes in the brain that resulted from the midazolam exposure and that could explain the behavioral deficits. Interestingly we did not detect overt differences between the higher and lower doses of midazolam exposure used in this manuscript, which raises the possibility that a threshold for damage exists even with lengthy exposure to modest doses.

Neurogenesis is ongoing at a very rapid rate in the hippocampal dentate gyrus in early postnatal life and then continues throughout adult life⁴⁵. The generation of new neurons in this brain region has been shown to be involved in certain types of learning in rodents⁴⁶ and the importance of adult neurogenesis in humans has been recently highlighted by data showing that deficits in hippocampal neurogenesis may be a feature of some forms of neurodevelopmental and psychiatric disease⁴⁷. Several investigations have suggested that exposure to GA can alter hippocampal neurogenesis, although it should be noted that relatively few studies have been done in this area and that no consensus can yet be reached from the available data. A single, long exposure to isoflurane at P7 has been found to result in an acute reduction in hippocampal neurogenesis⁹. The long-term consequences of this effect are uncertain, as the authors did not assay at an adult timepoint as we did in this study, but it is possible that an early reduction in neurogenesis could reduce the pool of neural precursor cells that are available later in life, thus reducing rates of neurogenesis. Zhu and colleagues examined the effect of three brief isoflurane exposures in early postnatal life on hippocampal neurogenesis at P45. They found both a shift towards glial cell fate in cells undergoing cell divisions at the time of the exposure and they also measured a decrease in a mitotic marker at P45⁴⁸, suggesting an ongoing deficit in neurogenesis that mirrors our findings with midazolam sedation. Early postnatal exposures to propofol on three sequential days at doses that could be considered consistent with either GA or sedation result in a reduction in neurogenesis measured 10 days later⁴⁹. By contrast Qiu and co-workers found that a short, single exposure to sevoflurane affected neither acute nor chronic neurogenesis and also had no influence on cell fate determination⁵⁰. Thus, the preponderance of data seems to support the conclusion that exposure to either anesthetic or sedative agents has the potential to cause a lasting suppression of hippocampal neurogenesis. Neither our data nor other studies show any clear evidence that this is the cause of deficits in hippocampal dependent learning, but this remains a very plausible hypothesis.

Electrochemical synapses are the site not only of neural transmission, but also of the plasticity that underlies cognitive functions such as learning and memory. Several investigators have found evidence that early developmental anesthetic exposure has disruptive effects on synapses. Lunardi and co-workers treated rats at P7 with a combination of anesthetic drugs including isoflurane, nitrous oxide, and midazolam and conducted electron microscopy in the neuropil of the hippocampal subiculum⁵¹. They measured a decrease in synaptic density in the hippocampal subiculum. These results are similar to those of Amrock and co-workers who found reduced synapse number in P100 and older rats that had been exposed to sevoflurane at P7⁵². They noted that 3 exposures of 2 hours each had a much more profound rate of synapse loss than a single exposure of 6 hours⁵². The finding that exposures spread over many days are particularly deleterious strongly suggests the possibility that an ICU sedation model which involves lengthy exposures for many days in a row could be particularly harmful. Not all studies of synapses in GA developmental toxicity have yielded uniform results. Briner and colleagues examined the effects of propofol on the pre-frontal cortex, and found that a single exposure to propofol has different effects on dendritic spines depending on the age at exposure. These spines, which are the post-synaptic elements of pre-frontal cortex, were decreased in rats exposed at P5, whereas P15 exposure caused an increase, leading the authors to conclude that the maturity of the neurons exposed

plays a key role in their response⁵³. By contrast, our own work in the mouse hippocampal dentate gyrus revealed reduced spine density in spite of exposures that occurred at P18⁴. In our sedation model we see a decrease in pre-synaptic labeling, which was accompanied by an increase in labeling with an excitatory post-synaptic marker that should correspond to the same post-synaptic elements that are on the spine. A reduced number of pre-synaptic elements indicates a reduced number of synapses, but the significance of the increase in excitatory post-synaptic elements is harder to interpret. One possibility is that proliferation of post-synaptic elements may develop after the loss of pre-synaptic elements as a compensatory mechanism. In any case, our findings strongly suggest that early developmental exposure to midazolam in a sedation model disrupts synapse formation.

Based on our findings, we strongly believe that further investigation into the putative phenomenon of developmental sedation toxicity is warranted. We were not able to model the type of continuous sedation that is often used in ICU settings in our *in vivo* mouse model, but we have no reason to think that continuous sedation is more benign than the chronic, repetitive exposure that we conducted. The use of the mouse as a model system has some caveats in this study, just as it does in studies of developmental anesthetic neurotoxicity. Physiologic monitoring in young rodents is challenging, and it is impossible for us to completely exclude the possibility of sedative effects on brain development that are mediated by hypoxia, hypotension, hypothermia, and/or hypercarbia rather than direct effects of the drug on the developing brain. Furthermore, the developmental timeline of a mouse is so short that the duration of exposure is much more significant as a total percentage of the time required for brain development. This limitation could be overcome by use of a large animal model such as a pig or a non-human primate, which would have the added benefit of removing the potential confounders of physiologic perturbation such as hypoxia and hypotension. Also, while we found that body weight did not differ substantially between control and experimental groups at P60, it is difficult to completely exclude a nutritional effect in the mouse model, for instance we did not measure blood glucose which could have an effect that is distinct from overall weight loss. Both experimental and control groups were separated from the dams, but it is possible that this separation has different consequences in sedated and un-sedated mice. Another limitation relates to our controls, which did not receive any injections, as we found that sham injections without sedation rapidly produced abnormal behaviors. Also, our culture model and *in vivo* model were conducted in different species and different brain regions. Finally, this study was conducted using only one sedative drug, and further work should establish whether the benzodiazepines are the only class of drugs given for pediatric ICU sedation that have potentially harmful effects on brain development. Our data also provide strong justification to conduct a retrospective study in humans to search for a correlation between ICU sedation and altered cognitive outcomes.

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Figure 1. Schematic representation of the experimental timeline *in vivo* and *in vitro*.

(A). *In vivo* paradigm: Body weight was assessed on the day before the midazolam treatment. Sedation with midazolam was conducted from P18 to P22. Y maze was tested on P60 and P61, fear conditioning was tested on P62 and P63. The immunohistochemistry for studies of synaptogenesis and neurogenesis was done using a separate set of animals. For studies of neurogenesis, BrdU injection occurred from P60 to P62. For studies of both synaptogenesis and neurogenesis (separate cohorts) tissue harvesting was at P63.

(B). *In vitro* paradigm: Dissociated cortical neurons in culture were treated with a range of doses of midazolam at 9am on 7DIV. Fresh media change with midazolam in solution was done to maintain the concentration of the midazolam in the media. The cells were fixed in 4% PFA on the 10DIV for immunocytochemistry.

Figure 2. Midazolam sedation impairs spatial learning and memory.

(A–B). In the Y-maze test (diagram in A), control animals exhibit normal performance, spending longer times in the novel arm. Midazolam sedated animals (both low and high dose groups) did not differ significantly in time spent in the novel and old arms, suggesting impaired learning and memory (B). (n=13 per group, * $p < 0.05$ t-test, n.s. indicates no significant difference)

(C–E). In the fear conditioning test (diagram in C,D), as compared to controls, the midazolam sedated mice (both low and high dose groups) showed reduced freezing time percentages in the contextual test (E). There was also a reduction in the freezing time percentage in the cued test (F). (n=13 per group. * $p < 0.05$, *** $p < 0.001$ ANOVA)

Figure 3. Midazolam sedation exposure decreases the long-term neuron proliferation. (A). Fig A shows representative immunofluorescence images of BrdU (green), a marker of cell proliferation, and NeuN (red), a neuron marker, in the dentate gyrus. The yellow arrowheads denote double labeled BrdU+NeuN+ cells. (Scale bar=100µm) (B). A representative example taken from the 20mg/kg image from A is shown in order to more clearly demonstrate double labeling of BrdU+NeuN+ that was quantified. (Scale bar=15µm) (C). Quantitative analysis showed a decrease in BrdU labeling in the midazolam groups compared to the controls in the neurons (n=18 per group, *p<0.05, ANOVA)

Figure 4. Midazolam sedation inhibits synaptogenesis in vivo.

(A). Figure A shows representative images of Synapsin-1 (green), a pre-synaptic marker, and Homer-1 (red), a post-synaptic marker in the dentate gyrus. The yellow arrowheads in the DAPI figure denote the imaged locations of the magnified synaptic markers in green, red, and merged channels below. There is a decrease of double labeled puncta (showed as the blue arrows in A) in the midazolam groups compared to the control (B). There is a decreased puncta number and fluorescence intensity of Synapsin-1 labeling in the midazolam sedated groups (both low and high dose) relative to controls (C-D). There is an increase in Homer-1 puncta number and labeling intensity in the midazolam groups (both low and high dose) relative to the controls (E-F). (n=27 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ANOVA, n.s. indicates no significant difference).

Figure 5. Midazolam sedation inhibits synaptogenesis in vitro

Representative images of Synapsin-1/ Homer-1 (green), Map-2 (red), DAPI (blue) immunofluorescence in neurons in dissociated culture at 10DIV are shown (A). The yellow arrowhead in the figure denotes the imaged locations of the magnified synaptic markers in green, red, and merged channels below. 50nM midazolam exposure resulted in no significant change in the both puncta number and fluorescence intensity of Synapsin-1 labeling, while 100nM and 150nM midazolam treatment groups showed a decrease in both the puncta number and intensity compared to the control group (B-C). 50nM midazolam exposure resulted in no significant change in the puncta number and fluorescence intensity of Homer-1 labeling, while 100nM and 150nM midazolam treatment groups showed an increase in intensity compared to the control group (D-E). (n=30 per group, *p<0.05, **p<0.01 ANOVA, n.s. indicates no significant difference)

Table 1.

Physiologic data during midazolam sedation

Group	Oxyhemoglobin saturation (%)	Heart rate (bpm)	Temperature (°C)
10mg/kg	95.65±2.61	473.33±19.22	36.37±0.24
20mg/kg	93.62±0.53	457.5±13.95	36.05±0.35

Table 2.**Sedation measurement scales**

The first component is a posture scale: A score of 0 is given for normal posture, feeding and grooming. A score of 1 is given for a mild decrease in muscle tone with reduced feeding and grooming behavior. A score of 2 is given for severe ataxia. A score of 3 is given for the presence of muscle tone but no movement. A score of 4 is given for no muscle tone and no movement. The second component is the righting reflex: This is assessed by placing a mouse on its back and observing the response. A score of 0 is given if it cannot be placed on its back. A score of 1 is given if it can be placed on its back, but rights itself immediately. A score of 2 if the mouse rights itself after multiple attempts. A score of 3 if the mouse attempts to right itself but cannot. A score of 4 is given if the mouse does not move at all in response to being placed on its back. The third component is corneal reflex scale: The animal's eyes are gently stimulated with a puff of air using a Pasteur pipette with a silicone nipple held 1 cm from its eyes: A score of 0 is given if the mouse moves its eyelids and a score of 1 is given if the mouse does not move its eyelids. The fourth component is a tail reflex: The mouse's tail is gently and suddenly pinched with blunt forceps. A score of 0 is given if the mouse moves its tail and a score of 1 is given if the tail remains motionless.

Posture scale	
0	Normal posture, feeding and grooming.
1	A mild decrease in muscle tone, reduced feeding and grooming.
2	Severe ataxia.
3	Weak muscle tone and no movement.
4	No muscle tone and no movement
Righting reflex scale Placing a mouse on its back and observing the response.	
0	Cannot be replaced on its back.
1	Can be placed on its back, but it rights itself immediately.
2	Rights itself after multiple attempts.
3	Attempts to right itself but cannot make it.
4	Does not move at all in response to being placed on its back.
Corneal reflex scale The animal's eyes are gently stimulated with a puff of air using a pasteurized pipette with a silicone nipple held 1 cm from its eyes.	
0	Moves its eyelids.
1	Does not move its eyelids.
Tail reflex scale The mouse's tail is gently and suddenly pinched with blunt forceps.	
0	Moves its tail.
1	The tail remains motionless.
Total scale score	0–10

Table 3.
Scale results of different doses of midazolam during the sedation time

Results for the sedation scale are presented for naïve control, low dose (10mg/kg), and high dose groups (20mg/kg). All scores were zero for controls, the low dose group resulted in moderate levels of sedation and the high dose group resulted in deep sedation.

	posture	righting reflex	corneal reflex	tail reflex	Total score
Control	0	0	0	0	0
10mg/kg	1–2	0–1	0	0	2–3
20mg/kg	2–3	2–3	1	0	5–7