Review Article

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Sedation with midazolam in the NICU: implications on neurodevelopment

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Abstract: The developing brain, particularly in premature infants, is highly susceptible to environmental and pharmacological influences. Premature neonates often require prolonged stays in the NICU, where midazolam (MDZ), a benzodiazepine, is commonly used as a sedative, despite concerns raised by the FDA in 2016 regarding its potential neurological complications in infants. Understanding the long-term effects of MDZ on these vulnerable patients is hindered by ethical considerations and limited research. This review emphasizes the vulnerability of premature infants to sedation and anesthesia and outlines how early exposure to MDZ can impact brain development at both molecular and behavioral levels, drawing from clinical and preclinical data. Additionally, we highlighted existing knowledge gaps and suggested avenues for further research to better comprehend the enduring consequences of MDZ exposure on neurodevelopment in this population.

Keywords: midazolam; benzodiazepine; sedative; NICU; neurodevelopment

Introduction

In the Neonatal Intensive Care Unit (NICU), where medication choices are constantly evolving, one major concern for caregivers is the early exposure of neonates to anesthesia and sedatives. This is particularly crucial given the vulnerability of neonates during the critical period of brain development.

Midazolam (MDZ), the most used sedative for neonates approved by the FDA, is widely administered in the NICU [1, 2]. Initially considered a safe sedative, recent research since 2003 has raised concerns about its neural complications.

This review aims to present current evidence on the effects of MDZ, alone or in combination with other anesthetics, on the neurodevelopment of animals and humans. We will begin by briefly outlining the mechanism, pharmacodynamics, and pharmacokinetics of MDZ. Subsequently, we will delve into the effects of MDZ on children, encompassing both preclinical and clinical evidence of altered behavior. Following this, we will explore the impact of MDZ on neural biology systems, including neuronal, glial, dendritic, and synaptic functions. Finally, we will discuss existing limitations and propose areas for future research.

The vulnerabilities of premature brain

Preterm infant is defined as an infant born prior to 37 weeks' gestation. Such infants are categorized based on their gestational age as extremely preterm (<28 weeks), very preterm (28–31 weeks), moderately preterm (32–33 weeks), and late preterm (34–36 weeks) [3]. In 2022, approximately 10 % of infants were born preterm, with a rise by 4 % in the 2020–2021 period [4]. During pregnancy, the final months and weeks play a crucial role in the development of vital organs such as the brain, lungs, and liver. Babies born prematurely, especially before 32 weeks, exhibit higher rates of mortality and disability [5]. In 2020, preterm birth and low birth weight accounted for about 16 % of infant deaths (deaths before 1 year). Surviving neonates may have breathing problems, feeding difficulties, cerebral palsy, developmental delay, vision problems, and hearing problems [4].

The Neonatal Intensive Care Unit (NICU) is a pivotal environment for managing and providing essential care to preterm infants, with the duration of stay varying based on factors such as birth weight, gestational age, and maternal complications. Notably, infants weighing less than 1,000 g

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may spend an average of 79 days [6] in the NICU, whereas full-term infants typically have a mean stay of 10 days [7]. Prolonged NICU stays are influenced by factors like growth retardation, infections, feeding issues, and respiratory complications [7–10].

The last trimester period of development is crucial for normal brain development. During this period, the human brain undergoes significant growth, doubling in size by 6 months and tripling by 12 months after birth [11–13]. Tremendous changes can occur even before birth. Neuroimaging studies reveal substantial differences in brain structure between 28 and 38 weeks of gestation, emphasizing the intricate nature of early brain development [11–13]. Specifically, at the 28th week of gestation, the brain is small and simple, with less folding than the brain of full-term infants (at the 38th week of gestation). The speed and complexity of the neurobiological processes governing early brain development place them at higher risk of developmental adversity.

Neurobiological processes, including neurogenesis, neuron migration, neuroapoptosis, synapse proliferation, pruning, and myelination, shape the developing brain [14–16]. The process of neurogenesis begins in the early stages of gestation following conception, with neuron migration occurring between the 12th and 20th weeks of gestation, facilitated by the development in the germinal matrix and subventricular zone [15]. Subsequently, cortical neurons navigate along a scaffold of glial cells to reach their final destinations. Following the migration, neuroapoptosis, characterized by programmed cell death, becomes notably active from 24 weeks of gestation until approximately 4 weeks after birth [14]. An additional crucial neurodevelopmental phase involves the proliferation of neuronal synapses, beginning around the 20th week of gestation and progressing rapidly. Synapse numbers peak at approximately 1–2 years of age, with figures 50 % higher than those observed in adults [14, 17, 18]. Following this peak, certain synapses undergo pruning, eliminating “unneeded” neurons, resulting in the loss of synaptic connections. The fourth major neurodevelopmental process, myelination, initiates towards the end of the second trimester and continues more gradually throughout childhood.

Notably, neonates in the NICU also have a high incidence of neonatal brain injuries. Some common brain injuries that preterm infants often encounter are intraventricular hemorrhage (IVH) or periventricular leukomalacia (PVL) [19]. IVH, a leading cause of brain damage in preterm newborns, occurs when the germinal matrix and subventricular zone sustain injury [19]. This vulnerability arises from the immaturity of blood vessels in the highly vascular germinal matrix, coupled with poor tissue vascular support, making this region especially prone to IVH [19, 20]. PVL is another important brain lesion that affects preterm newborns, which further can cause cerebral palsy and cognitive, behavioral, and attention deficits [19]. This type of injury specifically affects the cerebral white matter, resulting in decreased volumes in areas like the cortex, thalamus, and basal ganglia [19]. The lesion typically begins as a focal point in deep periventricular white matter zones due to neuronal necrosis, reactive gliosis, and microglial activation [19].

Given the complexities of these developmental phases, exposure to chemicals, including anesthetics and sedatives, may disrupt the normal morphogenesis of brain structures, leading to significant long-term damage. The heightened activity during synaptogenesis, synaptic pruning, and myelination – referred to as the brain’s growth spurt – in the second and third trimesters makes the fetal brain highly sensitive to anesthetic and sedative agents [21, 22]. Premature infants, often born during or before this growth spurt, are particularly vulnerable to the neurotoxic impacts of these drugs, potentially resulting in enduring cognitive impairment [21–24]. The underdeveloped state of neonatal organ systems, including the central nervous system (CNS), renders premature infants more prone to the depressant effects of these drugs, potentially inducing cerebral hypoperfusion and metabolic disruptions [23]. While the specific molecular mechanisms underlying this vulnerability are not yet fully understood [24], factors such as the immaturity of neurotransmitter systems and the delicate balance of neurochemical processes during synaptogenesis contribute to the heightened sensitivity of the premature brain to anesthetic neurotoxicity. The debated clinical significance arises as some children exposed to multiple and prolonged anesthesia report deficits larger than those with single exposures [25–28]. Further investigation is essential to elucidate the connection between exposure and brain cell loss and to formulate protective strategies.

Overview of midazolam

Among all benzodiazepines (BDZ), MDZ is the preferred choice for inducing sedation in neonates, primarily due to its rapid onset of action and shorter half-life. MDZ is the first-identified water-soluble BDZ, featuring an imidazole ring fused to a benzene ring integrated with a seven-membered diazepine ring. MDZ structure is unique in a way that the environmental pH-dependent ring-opening phenomenon characterizes its property. MDZ is highly water-soluble in a mild acidic environment (pH<4.0). Once the pH increases to physiological pH, the MDZ’s imidazole ring

closes, which makes it much more lipid soluble. This unique structural characteristic is a primary contributor to making MDZ accessible to the blood-brain barrier and executing the rapid onset action of the mechanism [29–31]. A summary of MDZ structure, pharmacokinetics, and pharmacodynamics is presented in Figure 1.

While the pharmacodynamics of MDZ in preterm neonates have not been thoroughly investigated, it is important to note that there is currently no established correlation between MDZ concentration and clinical effects in term neonates or children [32, 33]. Nevertheless, in the NICU, MDZ is often used in combination with opioids (fentanyl, morphine, and diamorphine) for ventilated neonates [2, 34]. Notably, endotracheal intubation and mechanical ventilation are major components of routine intensive care for very low birthweight and critically ill full-term infants [2, 34]. MDZ can provide anxiolytics, sedation, muscle relaxants, anterograde amnesia, and anticonvulsant effects by binding on γ2 subunit of the gamma-aminobutyric acid alpha ($GABA_A$) receptors. Once bound, MDZ modulates the activation of the $GABA_A$ receptors, which increases the frequency of opening of chloride channels that subsequently leads to the influx of Cl^- into the cell membrane, which results in membrane hyperpolarization and neuronal inhibition [29–31].

Approximately 97 % of MDZ is bound to plasma protein, primarily albumin, in adults and pediatric patients. Metabolized in the liver through cytochrome P450-3A family (CYP3A) enzymes, particularly CYP3A4 and CYP3A5, MDZ undergoes hydroxylation as the main metabolic pathway. This results in the formation of the active metabolite alpha-1 hydroxy midazolam (about 60–70 % of biotransformation products), 4-hydroxy-midazolam (5 %), or dihydroxy derivative [35, 36]. Following hydroxylation, glucuronidation occurs, a process in which glucuronic acid is attached to the metabolite, enhancing its water solubility and facilitating elimination via urine.

Neonates can perceive pain when undergoing invasive procedures such as surgeries, mechanical ventilation, and intubation, which can then induce agitation in newborns

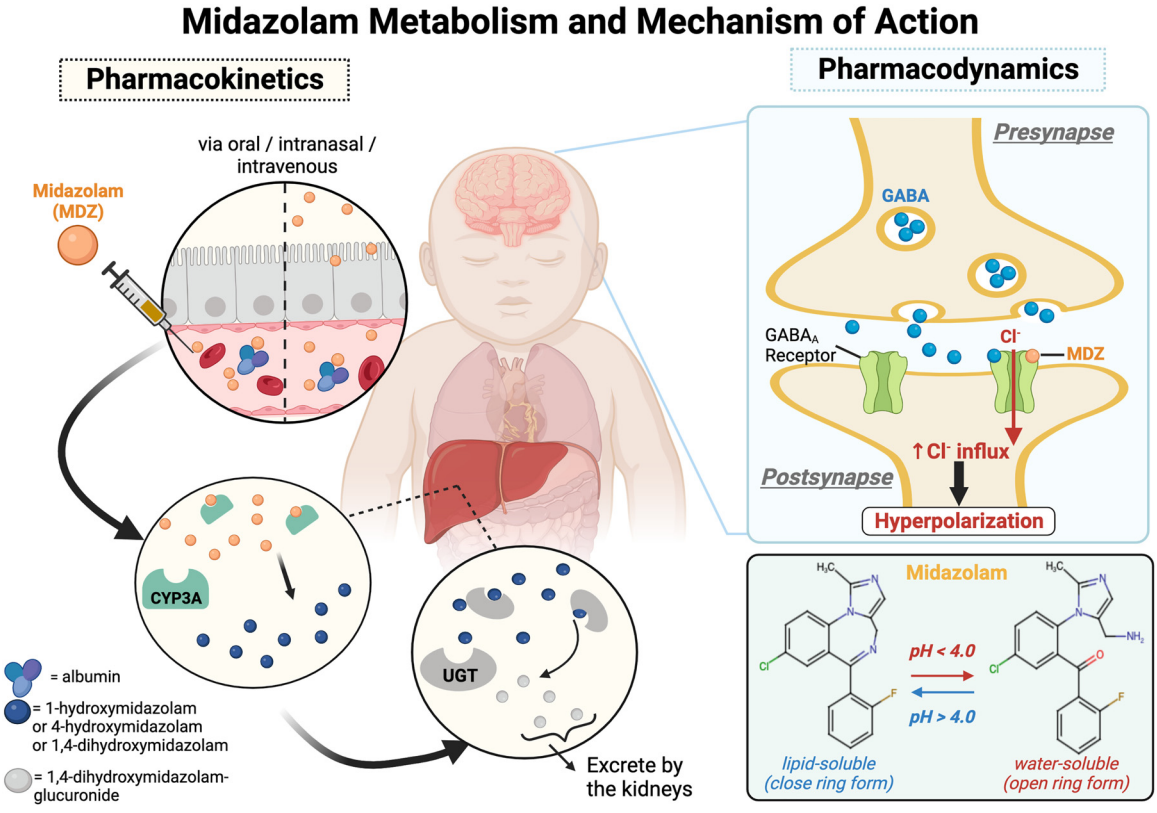

Figure 1: Overview of midazolam metabolism and mechanism of action. Midazolam (MDZ) can be administered orally, intranasally, or intravenously. Once MDZ is in the bloodstream, it can be bound to albumin and transported to either the brain, where it induces its mechanism of action, or to the liver to be converted to the metabolites formed by CYP3A enzymes and later excreted by the kidneys. In the brain, MDZ bound allosterically to the $GABA_A$ receptor, which promotes the gamma-aminobutyric acid (GABA) binding to the receptors, which increases the influx of chloride ions (Cl^-) into the postsynaptic cell. The Cl^- influx increases the negativity of the cell potential, subsequently leading to hyperpolarization. The figure is created by Biorender.com.

[37]. In the clinical setting, the optimal dose of MDZ is varied due to clinical situations and factors such as the patient's age, coexisting medical conditions, and concomitant medications. Pacifici wrote a review on the clinical pharmacology of MDZ in neonates and children, which also reviewed the dosages of MDZ in clinical settings [2]. The neonates and infant patients' ages can vary between less than 28 weeks of gestation to 8 years of life [2]. Depending on health conditions, age, and procedure requirements, MDZ can be administered as bolus injections or followed by continuous infusion.

The adverse effects of MDZ or BDZs used on neonates are debatable since some studies did not note any adverse outcomes, while others did [2, 32, 38–42]. Life-threatening adverse effects of MDZ administration are related to respiratory depression, hypotension, cardiac arrest, decreased blood flow, and increased brain injuries such as IVH and PVL [43, 44]. Other major adverse effects associated with long-term MDZ administration are tolerance development, withdrawal symptoms, and delayed awakening [2, 32, 38–42]. In addition, The Neonatal Outcome and Prolonged Analgesia in Neonate (NOPAIN) trial has shown that MDZ is associated with poor short-term neurologic outcomes with about 33 % of MDZ-exposed neonates affected by phase III of IVH, PVL, and death, which is much higher when comparing to placebo or morphine cohort [43].

Numerous animal studies compellingly demonstrate that general anesthetics and sedative medications elicit diverse morpho-functional changes throughout brain development [45]. For MDZ exposure or related MDZ-anesthesia models, the rodent model is often the first choice for the preclinical model. Though lacking nonhuman primates (NHP) model data in early MDZ exposure model, rodent models of MDZ found similar apoptotic injury patterns as NHP exposed to other anesthetics agents such as ketamine and can offer several advantages given the cost and ethical concerns of studies using NHP. The MDZ preclinical models are also varied but can be categorized into 2 groups: anesthesia (single exposure in only 1 day) and sedation (multiple exposures or multiple days) models. For the preclinical model, rodents are often used. In the anesthesia model, MDZ is often co-administered with anesthetic agents such as isoflurane and nitrous oxide (N_2O) for 6 h (Tables 1 and 2). Meanwhile, in the sedation model, MDZ is often treated alone and can be repeated from 3 to 18 days. Tables 1 and 2 present the age of the rodent animals, often starting on postnatal day 3 or day 7 (rats) and P10–P18 (mice). Most of the available rodents often start the treatment on P7, which is considered a peak in synaptogenesis in rodents. Very few *in vitro* models often use cortical primary neurons or embryonic hippocampi with dose ranges of 50–150 nM [46, 47] up to 30 mM [48].

Midazolam exposure during childhood: effects on neurobehavioral outcomes

Young animals and children are particularly vulnerable to the effects of anesthetics and sedatives, a topic extensively explored in the literature [69]. While challenges persist in translating findings from animal studies to humans, concerns about alterations in brain systems and subsequent changes in behavioral states are significant, especially when indicators are not obvious. In this part, we will comprehensively evaluate animal and human data, delving into the nuanced landscape of behavioral changes resulting from exposure to MDZ.

Anesthetics and sedatives during early development have been linked to behavioral changes, as observed in rodent models [69]. In 2009, Wilder et al. conducted a pioneering large-scale retrospective study on anesthetic exposure in individuals under 4 years of age, examining the incidence of later behavioral, learning, or developmental issues during school age [25]. The findings revealed that subjects with prolonged or repeated exposure to anesthesia exhibited an approximately two-fold increase in the identification rate of such problems compared to those without exposure. Since then, various retrospective studies have been published, yielding mixed results concerning the association between anesthetic exposure and subsequent neurobehavioral and developmental problems [70, 71]. Yet, the precise age at which the human brain undergoes its most vulnerable phase to the neurotoxic impacts of anesthetic and sedative drugs remains unclear and contentious. This uncertainty arises as there is a limited endorsement for postponing essential procedures in young children to minimize neurodevelopmental risks. In addition, not only direct exposure to anesthesia but also exposure to anesthesia via maternal also affects behavior in fetal and newborn rats. For instance, multiple works have shown that *in utero* or prenatal exposure to opioids such as morphine or oxycodone could induce heightened social anxiety and declined cognitive scores [72, 73].

Many available clinical studies consistently indicate an intensified risk of learning and behavioral disabilities in individuals with early exposure to general anesthesia and sedative agents [69, 71, 74–77]. While ample evidence explores the impact of opioids, such as fentanyl and morphine, on learning abilities and cognitive functions, limited

Table 1: Behavioral alterations post-midazolam exposure in young animals.

Species (postnatal day, P)	Treatment		Study significances	Ref.
	Components	Dosage and duration		
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 % for 6 h	Impaired spatial reference memories capabilities at adolescence and adulthood	[22]
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 70 % for 6 h	Lasting influences on socio-emotional status in young adult rats (P63–87): – ↑ risk-taking tendencies and alterations in anxiety-related behaviors – ↑ novelty-seeking tendencies and less guarded-behavior	[49]
Sprague-Dawley rat (P7)	MDZ ± pre-treated minocycline (30 min before MDZ injection)	9 mg/kg ± 40 mg/kg	Adult rats showed low spatial learning in MDZ group. Pre-treated with minocycline improved spatial learning and memory in adulthood.	[50]
Sprague-Dawley rat (P3, 10, 21, and 40)	MDZ	0.1 mg/kg, 1.0 mg/kg, and 10 mg/kg	Neonates: ↓ reflex thresholds and ↑ reflex magnitudes Older rat pups and adults: ↑ reflex thresholds and ↓ reflex magnitude	[51]
C57BL/6J mice (P10 and P20)	MDZ	50 mg/kg	P20 mice: acute ↓ locomotor activity at 3 and 24 h post-exposure but not after 72 h no long-term effects on learning and memory behaviors in adult (P105)	[52]
C57BL/6 mice (P18–22)	MDZ	10 mg/kg and 20 mg/kg, maintained for 12 h per day, repeated for 5 consecutive days	Impaired spatial and learning memory in adult (P60–63): – No differences in exploration time between original and novel Y-maze arm – ↓ freezing time percentage of contextual and cued fear conditioning	[46]
Sprague-Dawley rats (P7)	MDZ	10, 25, or 40 mg/kg at 15 min prior to H/R	↓ learning and memory impairment in H/R-induced model	[53]
C57BL/6 mice (P7–9)	MDZ	10 mg/kg, repeated single dose, 3 days	12 w mice: – ↓ preference for an object placed at the NLR – ↓ contextual fear memory in FC Recovered cognitive ability post-voluntary exercise	[47]

Key: ↑, increased/elevated; ↓, decrease/reduced; FC, fear conditioning; H/R, hypoxia/reoxygenation; NLR, novel location recognition; P, postnatal day.

research focuses on the cognitive and behavioral outcomes of children exposed to MDZ despite it being a commonly chosen sedative for neonatal and childhood care.

Duerden et al. conducted studies on very preterm neonates, assessing the long-term effects of MDZ exposure. Magnetic resonance and diffusion tensor imaging scans revealed decreased hippocampal volumes and lower cognitive scores at 18 months and 8 years of corrected age [78, 79]. McGraw reported increased postoperative behavior changes in children given preoperative oral MDZ, indicating potential long-term effects beyond the immediate postoperative period [80]. Research by Puia-Dumitrescu et al. on extremely preterm infants exposed to opioids and/or benzodiazepines for more than 7 days highlighted lower cognitive, motor, and language scores at 2 years corrected age. This study emphasizes the potential cumulative impact of prolonged exposure to these sedatives on developmental trajectories, warranting further research to understand specific mechanisms and long-term consequences [81]. Furthermore, the implications of MDZ in pediatric cardiac surgery are noteworthy, with evidence indicating that the total MDZ dose may adversely affect neurodevelopmental outcomes in infants [82]. Additionally, the combined use of MDZ and phenobarbital, both GABAergic drugs, has been linked to increased neuronal injury in neonatal seizures [83]. These findings are particularly concerning given the potential long-term impact on neurodevelopment, especially in vulnerable populations such as preterm infants.

Table 2: Midazolam's effects on developing neurobiology in the *in vivo* and *in vitro* models.

Species (postnatal day, P)	Treatment		Brain regions	Study significances	Ref.
	Components	Dosage and duration			
Sprague Dawley rats (P7)	MDZ	3, 6, or 9 mg/kg	Whole brain	No increased in neuroapoptosis ↓ LTP in hippocampus slices at P29–35	[22]
	Isoflurane + MDZ	0.75 % + 9 mg/kg for 6 h	LD, AV thalamic nuclei, parietal cortex (layer II)	↑ neuroapoptosis	
	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 % for 6 h	Thalamus, parietal cortex, widespread effects in other regions	↑ neuroapoptosis ↓ LTP in hippocampus slices at P29–35	
Sprague Dawley rats (P1, 3, 7, 10 and 14)	MDZ	3, 6, or 9 mg/kg		No increased apoptosis	[54]
	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 %; for 2, 4, or 6 h	Posterior cingulate/ retrosplenial cortex, the parietal and occipital cortex, subiculum, anterior thalamus, amygdala	↑ neuroapoptosis (age-dependent) No disturbance in cortical cerebral blood flow, hypoxia, and hypoventilation Activated intrinsic apoptotic pathway: – alterations of bcl_{xL} – ↑ cytochrome C release and caspase-3, −8 and −9 activation	
Sprague Dawley rats (P7 and 17)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 % for 6 h	CC and anterior thalamus	↑ neuroapoptosis but alleviated when treated with melatonin	[55]
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 %; for 2, 4, or 6 h	CC and anterior thalamus	Disturbance in BDNF-activated apoptotic cascade (thalamus: ↓BDNF; CC: ↑BDNF) via Trk-dependent (thalamus) and Trk-independent $p75^{NTR}$-dependent (CC) pathways ↓neuroapotosis via β-estradiol application	[56]
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 % for 6 h	CC and anterior thalamus	Altered synaptic proteins level after anesthesia administration Induced permanent neuronal loss after 3–23 days post-anesthesia	[57]
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 75 % for 6 h	Subiculum	Impaired neuropil structure, ↓ MSBs at 2 weeks post-exposure ↓ number of synapses per μm No preferential loss of different types of synaptic contacts or excitatory vs. inhibitory synapses	[58]
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 70 % for 6 h	Hippocampus	↓ H3-acetylation in BDNF and c-Fos genes, manifested by CBP fragmentation ↓ BDNF and ↓c-Fos proteins: impaired neuronal development and synaptic neurotransmission	[59]
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 70 % for 6 h	Subiculum	Impairment of mitochondrial morphogenesis (↑ mitochondria area), structural integrity (↑ complex IV activity, ↓ regional distribution in presynaptic) ↑ autophagic activity and ↓ mitochondria density Disturbances in inhibitory synaptic neurotransmission	[60]

Table 2: (continued).

Species (postnatal day, P)	Treatment		Brain regions	Study significances	Ref.
	Components	Dosage and duration			
Sprague Dawley rats (P7)	Isoflurane + MDZ + N_2O	0.75 % + 9 mg/kg + 70 % for 6 h	Subiculum	30 % rise ROS and a 2-fold decline in superoxide dismutase. Disrupts mitochondrial dynamics by promoting excessive fission via dynamin-related protein 1 sequestration and mitofusin-2 downregulation.	[61]
C57BL6 mice (P7)	MDZ ± ketamine	9 mg/kg ± 40 mg/kg	CC, caudate putamen	↑ neuroapoptosis, but more robust when combined with ketamine	[62]
YFP-expressed mice (P15, 20, and 30)	MDZ	25 mg/kg, repeated for 3 times per 90 min intervals	Somatosensory cortex, hippocampus	↑ dendritic spine protrusion density and ↓ protrusions head width and lasts for several days	[48]
P6-P7 hippocampus slices	MDZ	30 μM, 5-hour-long bath	Hippocampus CA1 pyramidal neurons	↑ dendritic spine protrusion and ↑ formation density rapidly after treatment, and lasted up to 48 h ↑ spinogenesis	
GFP-M transgenic mice (P8–12)	MDZ	50 mg/kg, repeated single dose, 5 days	Hippocampus pyramidal neurons	↓ spine density and ↑ spine length of hippocampal CA1 pyramidal neurons at P13 but not P30	[63]
E19 Wistar rats	MDZ	100 and 300 nM on 15 DIV	Hippocampus	↑ neuronal apoptosis ↑ cytosolic Ca2+-concentration Suppresses neuronal Ca2+-oscillations amplitude and frequency (dose dependent) ↓ synapses number after 24 h	[64]
Wistar rats (P5 and P15)	MDZ	Initially with 25 mg/kg then 12.5 mg/kg to achieve total of 6 h sedation; repeated up to 7 injection in a single day	mPFC	↑ neuroapoptosis without impacting total neurons and neuronal density at both early (P5) and later (P15) stages of brain development Altered GABAergic neuronal phenotype, with stage-dependent effects on PV and CR neurons	[65]
Sprague-Dawley rat (P7)	MDZ ± pre-treated minocycline (30 min before MDZ injection)	9 mg/kg ± 40 mg/kg	SVZ, SGZ of hippocampus, LV, DG	↓ cell proliferation SVZ and SGZ after 7 days ↑ cell proliferation when pre-treated with minocycline	[50]
C57BL/6 mice (P18–22)	MDZ	10 mg/kg and 20 mg/kg, maintained for 12 h per day, repeated for 5 consecutive days	Hippocampus, DG	↓ long-term neurons proliferations ↓ presynaptic and ↑ post-synaptic density	[46]
C57BL/6 mice (P18–22)	MDZ ± morphine	MDZ: 5–9 mg/kg, MDZ + morphine: 2.5–4 mg/kg morphine and 5–9 mg/kg MDZ; escalating dose, twice daily for 5 days (from P18 to 22)	Whole brain	MDZ only: ↑ S100B expression and 17–18.5 kDa MBP splicing isoform expressions MDZ + morphine: − 20 % ↑ presynaptic marker synaptophysin − ↓ % weight gained − ↑ drebrin levels	[66]

Table 2: (continued).

Species (postnatal day, P)	Treatment		Brain regions	Study significances	Ref.
	Components	Dosage and duration			
C57BL/6 mice (P7–9)	MDZ	10 mg/kg, repeated single dose, 3 days	Hippocampus, DG	↓ NSC proliferation and neurogenesis in the DG at both P10 and P38 Inhibits NSC activation and neurogenesis in the adult DG but recover post voluntary exercise	[47]
Sprague-Dawley rats (P3–P21)	MDZ	1 mg/kg to 10 mg/kg; repeated single dose, escalate from P3 to P21	Cortex	Altered synaptic proteome GO analysis associated with actin binding, cytochrome c-oxidase, TCA cycle, etc. Validated ↑ ADD1 protein expression in MDZ synaptosome	[67]
Sprague-Dawley rats (P3–P21)	MDZ	1 mg/kg to 10 mg/kg; repeated single dose, escalate from P3 to P21	Cortex	Altered BDEV proteome GO analysis associated with sodium channel activity, adenylate cyclase, corticosteroid receptor signaling pathway, etc. Validated ↓ YWHAH post-MDZ exposure as BDEV marker	[68]

Key: ↑, increased/elevated; ↓, decrease/reduced; ADD1, alpha adducin protein; AV, anterodorsal; BDEV, brain-derived extracellular vesicle; BDNF, brain-derived neurotrophic factors; CBP, CREB-binding protein; CC, cerebral cortex; CR, calretinin; DG, dentate gyrus; DIV, days *in vitro*; EAAT2, Excitatory amino acid transporter 2; GO, gene ontology; H/R, hypoxia/reoxygenation; LD, lateral dorsal; LTP, long-term potentiation; MBP, myelin basic protein; mPFC, medial prefrontal cortex; MSB, multiple synaptic boutons; NSC, neural stem cell; P, postnatal day; PV, Parvalbumin; ROS, reactive oxygen species; SGZ, subgranular zone; SVZ, subventricular zone; S100B, calcium binding S 100 protein; YWHAH, 14-3-3 eta protein.

While clinical data on neonatal exposure to MDZ is limited, preclinical investigations suggest potentially harmful neurodevelopmental outcomes. The 'midazolam effect,' observed in animal studies, reveals a dose-dependent impact on reflex thresholds and behavioral changes. Seminal research by Jevtovic-Tedorovic et al. demonstrated that postnatal day 7 rats, continuously exposed for 6 h to a general anesthesia 'triple cocktail' including isoflurane, nitrous oxide (N_2O), and MDZ, exhibited lasting cognitive, behavioral, and memory deficiencies [22]. Another study found that MDZ administration in neonatal rats produced a dose-dependent reduction in mechanical reflex thresholds and a concurrent increase in both mechanical and thermal reflex magnitudes [51]. Conversely, in older rat pups and adults, MDZ produced the opposite outcome, elevating reflex thresholds and diminishing reflex magnitude [51]. Beyond traditional measures of intelligence and cognitive outcomes, researchers are expanding their focus to include behavioral testing and examining changes in anxiety and social interactions. For instance, one study by Diana et al. demonstrated that early exposure to a triple anesthetic cocktail similar to Jevtovic-Tedorovic's study led to altered behavior in the open field and elevated plus maze tests, suggesting increased risk-taking and anxiety [49]. Additionally, a study by Xu et al. found that repeated, long-duration exposure to MDZ can result in deficits in performance on the Ymaze and fear conditioning testing in young adult mice [46]. A summary of the effects of early MDZ exposure on behavior is provided in Table 1.

Both clinical and preclinical studies presented herein reveal that exposure to MDZ, particularly in vulnerable populations such as preterm infants and young children, can lead to lasting cognitive and behavioral deficits. Despite mixed findings and ongoing debates about the critical periods of vulnerability, the evidence indicates the necessity for cautious use of MDZ in specific patient groups. Further investigation is warranted to elucidate the mechanisms underlying these effects and develop strategies to mitigate potential long-term harm.

Effects of MDZ on neurobiology

Effects on neurons

Neurons are fundamental functional units of the nervous system responsible for controlling signal transmission and informational processing throughout the body. During the critical period of rapid synaptogenesis, the developing brain is particularly vulnerable to anesthetic agents [22, 54, 57, 69, 84, 85]. In rodents, this susceptibility peaks around postnatal day 7, while in humans, rapid synaptogenesis extends from mid-fetal gestation to several years after birth [14].

Preclinical studies have demonstrated that MDZ is associated with disruption in synaptogenesis and alterations in neuronal structure and function. Specifically, rodents exposed to MDZ followed by isoflurane and N_2O have been associated with increased neuroapoptosis [22, 54–57], activation of intrinsic apoptotic pathway [56], long-term hippocampal potentiation suppression [54], decreased synapse density [58], and impaired synaptic mitochondria structure and dynamic [60, 61]. A single exposure to only MDZ is debatable since some studies observed no neuroapoptosis [22, 62] while others presented increased neuronal cell death [62, 64] and were even robust when co-treated with ketamine [62]. Moreover, *in vitro* and *in vivo* studies also showed that exposure to MDZ can reduce neuronal proliferation [46, 50]. Repeated and prolonged exposure to MDZ during the neonatal period also led to enduring alterations in neuronal structure and function and can persist long-term. Studies employing prolonged MDZ sedation revealed significant dose-dependent changes in synaptic morphology (i.e., alterations in dendritic spine protrusion and density) and number of connections [48, 63, 64], reduced hippocampal neurogenesis, cell proliferation, and impaired learning and memory in rodents [46, 47, 50]. In addition, repeated exposure to MDZ at both early and later stages of development can also alter medial prefrontal cortex (mPFC) GABAergic neuronal phenotypes with a direct impact on parvalbumin and calretinin interneurons [65]. Giri and colleagues also showed that a single MDZ exposure could inhibit cell proliferation in the neurogenic regions of neonatal rats, suggesting potential impairment of neurogenesis during neonatal MDZ exposure. Interestingly, in the same study, the adverse effects on neurogenesis were reversed when neonate rats were treated with minocycline, a long-acting tetracycline agent with neuroprotective and anti-inflammatory properties, before MDZ injection [50]. Similarly, Doi et al. found that repetitive neonatal MDZ exposure negatively affects neurogenesis and cognitive ability, yet these effects were mitigated by voluntary exercise in adulthood [47]. Whether combining MDZ with other known neuroprotective agents or implementing exercise at a later developmental stage could mitigate the harmful effects of neonatal MDZ exposure remains to be further investigated.

Effects on glial cells

While considerable attention is given to neuronal impacts, it is essential not to disregard the influence of MDZ on glial function. Glial cells (i.e., astrocytes, microglia, and oligodendrocytes) play a pivotal role in the development and maturation of neurons. The emerging field of research underscores the contributions of glial to neural function and behaviors, including learning and memory [86, 87]. However, limited information is available on how early exposure to MDZ affects glial cells. Iqbal O'meara et al. demonstrated that repeated MDZ exposure in young mice significantly modulates the expression of specific myelin basic protein isoforms and S100B calcium-binding protein in the developing brain [66]. Another study by Tanabe et al., while not specifically focused on neonates or young animals, suggested that MDZ is associated with reducing cerebral endothelial ICAM-1 expression and suppressing IL-1β-induced IL-6 release from rat glial cells [88]. Alterations in acetylcholinesterase and butyrylcholinesterase activities further contribute to the intricate relationship between MDZ and glial dynamics.

Despite the uncertainties about MDZ's influence on developing glial cells, studies on other $GABA_A$ agonist sedatives and anesthetics with similar mechanisms of action (e.g., propofol, sevoflurane, desoflurane) indicate potential toxic effects on glial cells, particularly astrocytes, oligodendrocytes, and microglia. *In vitro* and *in vivo* studies on isoflurane revealed the toxic effects of isoflurane on impairments of cytoskeletal structure and organization during astrocyte development and maturation [89–92]. Additionally, a 3-h exposure to propofol in rat dentate gyrus nascent cells significantly increased astrocyte expression 24 h post-exposure [92]. Furthermore, 2 h of exposure to 3 % sevoflurane in P6 mice repeatedly at multiple time points was associated with microglia activation in the hippocampus [93].

Current evidence also suggests that the impairment in brain and cognitive function may be due to the negative impacts that anesthesia exposure had on glial cell architecture and modulated neuroimmune responses [94–98]. Anesthetics and sedative agents can exert both anti- and pro-inflammatory effects by modulating microglial activation, suggesting that anesthetics may play dual roles in cognitive impairments. Several studies have highlighted the

effects of BDZs on modulating neuroimmune responses in adult *in vivo* or *in vitro* models [99–103]. A recent study by Shi et al. suggested that chronic exposure to diazepam (DZP), a common sedative BDZ, induces cognitive deficits in adult mice via the mitochondrial 18 kDa translocator protein (TSPO) by altering microglial morphology and impairing synaptic material phagocytosis. These findings highlight a mechanism by which TSPO ligands modulate synaptic plasticity and consequentially lead to cognitive impairment [100]. TSPO, previously known as the peripheral BDZ receptor, is a transmembrane protein expressed ubiquitously in peripheral tissues and binds to BDZs. Although TSPO has been implicated in multiple biological functions, including steroid biosynthesis, apoptosis, and cell proliferation, its exact mechanism in regulating immune response remains ambiguous [104]. Some studies suggest that TSPO signaling can reduce pro-inflammatory cytokine production in microglia [99, 101, 105]. For instance, Horiguchi and colleagues demonstrated that MDZ exerts anti-inflammatory effects on LPS-stimulated macrophages by suppressing NF-κB/AP-1 and MAPK activation through TSPO mediation. This suppression reduces the activation of pro-inflammatory genes, including those encoding CD80, IL-6, and TNF-α [99]. The authors suggested that such interference with macrophage activation may be linked to the adverse side effects seen in some critically ill patients following MDZ administration [106, 107]. Wilms et al. also suggested that these BZDs may hinder microglial proliferation and mitigate inflammatory responses associated with activated microglia [101].

Molecular correlates

The brain is a complex network of interconnected circuits responsible for specific functions. Neurons communicate by releasing neurotransmitters into the synapse – the gap between neurons. These neurotransmitters bind to receptors on the receiving neuron, influencing its activity. Transporters recycle neurotransmitters, regulating the signal between neurons. MDZ, like other BDZs, directly binds to the $GABA_A$ receptor, enhancing GABA binding and limiting excitatory signals. This action can induce dopamine release by disinhibiting interneurons and promoting the activity of dopaminergic neurons [108]. The complex impact of MDZ on neurotransmitters also involves the disruptions in cholinesterase homeostasis, suppression of neuronal Ca2+ oscillations, and inhibition of hippocampal long-term potentiation and learning through BDZ receptors [64, 109, 110].

Additionally, the microenvironment of the synapse plays a crucial role in controlling the synaptic activities. Nguyen et al. demonstrated alterations in the proteome of synaptosomes, isolated subcellular fractions of synaptic terminals, in early neonatal rats exposed to prolonged MDZ. Their findings revealed up- and downregulated differentially expressed proteins (DEPs) involved in various molecular functions and biological processes, such as actin-binding, cytochrome c oxidase, pyridoxal phosphate binding, protein depolymerization, tricarboxylic cycle, and central nervous system neuron development [67].

In addition to exploring neurochemistry and the proteome, current research highlights potential long-term changes in gene transcription and functional deficits in learning and behavior induced by anesthesia. These effects may be mediated through epigenetic modifications. Notably, the literature emphasizes the significance of histone modifications in neural development and brain function [111]. Preclinical studies underscore the involvement of cyclic-AMP-response element binding protein (CREB) signaling pathways in anesthesia-induced neurodegenerative changes [111–113]. CREB-binding protein (CBP), also known as CBP, is a transcription activator and co-activator of CREB. Additionally, CBP functions as a histone acetyltransferase (HAT), regulating histone acetylation through its intrinsic HAT domain. Exposure to a sedative dose of MDZ, followed by a combination of nitrous oxide and isoflurane for 6 h in P7 rat pups, leads to the fragmentation of CBP and a subsequent decrease in its HAT activity at 2 h or 24 h (at P8) post-exposure. This subsequently results in hypoacetylation of H3, causing down-regulation in the transcription and expression of BDNF and c-Fos [59]. The long-term epigenetic changes in rodents exposed to MDZ during their neonatal period have yet to be explored.

The evidence discussed herein suggests that MDZ and other BDZs may affect neurodevelopment through various mechanisms. These effects are not limited to neuronal damage, alterations in synaptic function, modulation of glial cell activity, disruption of immune responses, changes in neurotransmitter dynamics, and epigenetic modifications. Therefore, it is imperative to further investigate the mechanisms by which MDZ influences neurodevelopmental biology and to assess the long-term consequences of both acute and chronic early exposure. Although few studies have explored therapeutic interventions to mitigate the cognitive impairments or adverse effects of neonatal MDZ exposure, this area of research holds significant promise for clinical application. This knowledge is essential for developing safer anesthetic and sedation practices, particularly for vulnerable populations such as preterm infants and young children, to mitigate potential adverse outcomes and ensure proper neurodevelopment. Detailed impacts of MDZ exposure during early development on neurobiology are provided in

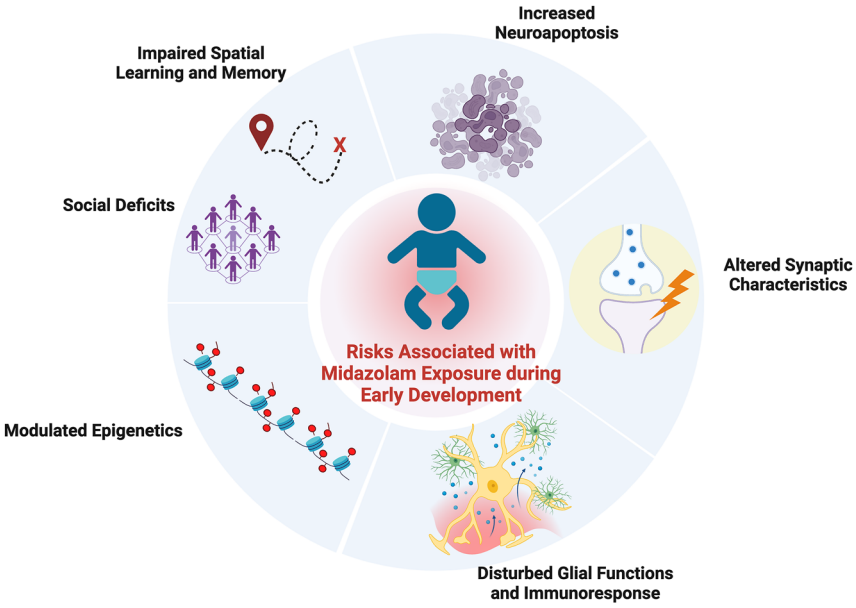

Figure 2: Overview of risks associated with exposure to midazolam during early development. Graphic summary of significant molecular, cellular, and behavioral deficits in neonatal exposure to MDZ. This figure is created by Biorender.com.

Table 2, and a summary of risks associated with MDZ exposure is described in Figure 2.

Future perspectives

The use of anesthesia and sedatives to prevent pain and manage agitation and stress has been a major advance in medicine and neonatal intensive care [37]. Despite the potential for limiting painful procedures, critically ill infants often undergo unavoidable stress-inducing interventions, such as intubation and catheterization. The utilization of analgesics (opioids and nonsteroidal anti-inflammatory drugs) and sedatives (benzodiazepines and other anesthetic agents) is widespread, but its application varies significantly across different hospital units. While the mechanism of action of many anesthetics, including MDZ, is still not completely understood, the potential for these agents to induce longer-term alterations in brain function, particularly in vulnerable populations such as premature neonates, warrants future investigation. Despite ongoing research on MDZ's potential longer-term effects, establishing a definitive link between general anesthesia/surgery/sedation and lasting behavioral or cognitive alterations in children remains debatable.

A significant gap in our understanding pertains to the cumulative impact of life stresses on the long-term effects of MDZ. Concerns persist, especially regarding the safety of prolonged MDZ use throughout developmental stages like early childhood, adolescence, and adulthood. Unfortunately, preclinical data is lacking for these specific time points. In addition, clinical studies emphasize that a considerable number of children undergo multiple and prolonged exposures to anesthetics and sedatives, resulting in reported deficits larger than those observed with a single exposure [25, 26, 28, 81]. The current preclinical model predominantly examines a single exposure, with very few models exploring the impact of multiple MDZ exposures, leaving uncertainties about the consequences of multiple exposures on molecular, cellular, and behavioral aspects.

The process of synaptogenesis is profoundly affected by MDZ alone or in combination with other anesthetic agents (Table 2). However, synapses are also regulated by intricate networks of proteins and signaling molecules that modulate the synaptogenesis process. Therefore, identifying biomarkers indicating abnormalities associated with MDZ exposure can offer insights into potential injury and toxicity-induced mechanisms. Biomarkers can aid in examining proposed preclinical mechanisms and facilitate translation between human and animal models. Additionally, since synapses house numerous mitochondria, it is crucial to acknowledge the potential impact on synaptic mitochondrial functions and the expression of mitochondria-associated proteins. Notably, growth factors acting as signaling molecules can directly or indirectly influence various biological processes, including synaptogenesis and synaptic pruning. However, there is limited data on the impact of chronic MDZ exposure during childhood on growth factor profiles.

Despite recent advancements in understanding the potential impact of MDZ on synapses, there remains a need for a more comprehensive exploration of the long-term effects of MDZ exposure on synaptic functionality. Some studies indicate that MDZ may decrease the long-term potentiation of synapses. Additionally, synaptic activities, such as neurotransmitter release and uptake, vesicle docking and recycling, and dendritic spine alterations, require substantial ATP, primarily regulated by mitochondria at pre- and post-synaptic sites. The scarcity of information on the short and long-term effects of MDZ on mitochondria and its influence on synaptic activities emphasizes the importance of understanding these aspects. Such knowledge can contribute to elucidating the mechanisms of neuroapoptosis and cellular dysfunction, thereby aiding the development of appropriate interventions and therapeutic strategies to address adverse effects associated with MDZ exposure in neonates and infants.

Conclusions

Midazolam, a widely used benzodiazepine sedative in the NICU, serves to alleviate agitation and induce sedation in neonates. Despite its effectiveness, its administration often accompanies invasive procedures such as surgeries and mechanical ventilation, contributing to various adverse outcomes encompassing respiratory, cardiovascular, and neurological domains. Recent studies have scrutinized the impact of single MDZ exposures on long-term outcomes, revealing adverse effects on cognitive function and behavioral deficits. This scrutiny extends to the molecular and cellular levels, where MDZ is found to induce apoptosis and alter cell morphology and functions. Although progress has been made in understanding baseline deficits, notable knowledge gaps persist. Particularly, there is a paucity of information on the consequences of prolonged/chronic MDZ exposures and their potential long-term effects on neurological and developmental outcomes. Addressing these gaps is crucial for advancing our understanding of MDZ's comprehensive impact in clinical settings.

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