Midazolam Potentiates Nociceptive Behavior, Sensitizes Cutaneous Reflexes, and Is Devoid of Sedative Action in Neonatal Rats

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Background: The significant postnatal maturation of γ-aminobutyric acid signaling in the developing brain is likely to have important implications for infant pain processing. γ-Aminobutyric acid receptor activation evokes analgesia and sedation in the adult, but the impact of immature γ-aminobutyric acid signaling on modulators of the γ-aminobutyric acid type A receptor, such as the benzodiazepines, is not known in infants.

Methods: Nociceptive processing was measured using behavioral and electrophysiological recordings of hind limb flexor withdrawal threshold and magnitude to mechanical and thermal stimulation of the hind paw. The effects of midazolam (0.1–10 mg/kg subcutaneously, 0.1 mg/kg intrathecally) or saline treatment were compared in rats aged 3, 10, 21, and 40 days (adult). The sedative action of midazolam was assessed at each age using righting reflex latencies.

Results: Midazolam dose-dependently decreased mechanical reflex thresholds and increased mechanical and thermal reflex magnitudes in neonates. In older rat pups and adults, midazolam had the reverse effect, increasing thresholds and decreasing reflex magnitude. These differences were mediated supraspinally; intrathecal administration of midazolam did not affect flexion reflexes at any age. Midazolam had no sedative action in the youngest rats; sedation increased gradually through postnatal development.

Conclusions: The results show a striking reversal in the effects of midazolam on nociception and sedation in rats between postnatal days 3 and 10. Midazolam fails to sedate young rats and sensitizes their flexor reflex activity. The sedative and desensitizing effects of midazolam are not observed until later in life after maturation in supraspinal centers. The results indicate a need to better understand the pharmacology of drugs used routinely in neonatal intensive care.

THE majority of synaptic inhibition in the adult central nervous system is mediated by the amino acid γ-aminobutyric acid (GABA) acting on postsynaptic GABA type A (GABA_A) receptors. Activation of this receptor allows the passage of Cl⁻ ions from the extracellular to the intracellular compartments, hyperpolarizing the cell and decreasing its excitability, which leads to various physiologic effects, including reducing nociception. This form of inhibition is, however, under powerful developmental regulation. GABA_A receptors undergo marked postnatal changes in the brain and spinal cord with respect to expression levels, distribution, and subunit composition. Furthermore, their physiologic function during development is influenced by high levels of neurosteroids and immature membrane transporter systems. In the late embryonic and early postnatal periods, activation of the GABA_A receptor in many parts of the central nervous system depolarizes rather than hyperpolarizes neurons because of high levels of intracellular Cl⁻ associated with low expression of the K⁺/Cl⁻ cotransporter type 2 protein.

The immaturity of inhibitory signaling is likely to play an important role in the shaping of spinal nociceptive networks during the early postnatal period in both animals and humans. This process can be characterized by the gradual reduction of dorsal horn cell receptive field size and increase in cutaneous thresholds and the gradual tuning of nociceptive reflexes. Maturation takes place over an extended postnatal period of several weeks in rats and is likely to be driven by the development of an appropriate balance between excitation and inhibition, not only in the dorsal horn but also at higher levels of the central nervous system. Postnatal maturation of GABA_A signaling and interneuronal pathways are likely to have practical consequences for the treatment of pediatric pain and anesthesia. Many clinically prescribed drugs, e.g., benzodiazepines, barbiturates, opioid analgesics and inhalation anesthetics, increase inhibition in the dorsal horn and other central nervous system regions either by direct action on the GABA_A receptor or via GABAergic interneuronal connections, yet the impact of immature inhibitory process-
ing on the efficacy and mechanism of action of these drugs remains unknown. We therefore performed a series of experiments to analyze the effects of the benzodiazepine midazolam, a positive allosteric modulator of the GABA<sub>\alpha</sub> receptor, on nociceptive processing throughout postnatal development. A dose-response study of midazolam on both mechanical and thermal nociceptive responses was performed using behavioral and electrophysiological techniques. Sedation levels were also monitored in these animals as another index of the efficacy of this drug in neonates. We show that, in contrast to its effects on adults, midazolam decreases mechanical and thermal nociceptive thresholds and is significantly less sedative in the neonate. The results indicate that these effects are mediated supraspinally.

**Materials and Methods**

All animal procedures were licensed by the UK Home Office (London, United Kingdom) and performed in accordance with the Animals (Scientific Procedures) Act 1986. Sprague-Dawley rats of either sex were used in all experiments. Animals were kept in cages with their littermates and mothers (where appropriate) in a room with a 12-h light/dark cycle.

**Behavioral Assessments of Mechanical Withdrawal Thresholds**

Mechanical thresholds were measured using von Frey hair filaments (Vfh; 0.01-300.0 g) (Stoelting, Wood Dale, IL) in 3-, 10-, 21-, and 40-d-old rats (postnatal day [P] P3, P10, P21, and P40, respectively, approximately corresponding to P3 = 28-wk gestation human fetus, P40 = adolescent human). Animals were habituated to the testing room for 10 min (P3), 20 min (P10), 30 min (P21), or 1 h (P40) before testing. Habituation times were determined by the time required for rats to become quiescent at each age group and to prevent extended periods after feeding in preweaned neonates. P40 rats were placed on a raised wire mesh grid and hind paw plantar thresholds measured; all other ages were placed on a cotton wool sheet overlaying a heated blanket (Cozec Cumnford; Buro Dean Appliances Limited, London, UK) and tested on the dorsum of both hind paws. Dorsum testing in these ages, and the electromyographic response was recorded. Threshold was determined as the minimal hair number needed to elicit a reflex withdrawal above normal baseline electrical activity of the animal; a total of four hairs were used for testing: two subthreshold hairs (the two hair numbers below threshold hair), one threshold hair, and one suprathreshold hair (the hair number above threshold). Each hair was presented three times in sequence on the plantar of the left hind paw before moving on to the next hair number from the lightest to the heaviest hair. After baseline measurements, animals were injected subcutaneously with midazolam (10 mg/kg) or saline and tested every 5 min for 1 h thereafter or, in the case of the P3 pups, every 10 min from 5 min postinjection to avoid excessive tissue damage.

Thermal withdrawal thresholds were determined using electromyographic measurements in P3 and P21 rats as described above (separate groups of animals were used). Reflex magnitude was measured in response to a 4-s stream of 55°C water applied to the plantar surface of the hind paw. Baseline electromyographic recordings...
were made before midazolam injection, subcutaneously. Animals were then left for 15 min, and electromyo-
graphic activity was rerecorded in response to a further 4-s 55°C water jet.

**Intrathecal Administration of Midazolam**
Intrathecal injections were also performed; animals were anesthetized as above and maintained at 1.5% halothane through a nose cone. Midazolam (Sigma, Poole, Dorset, UK) 0.1 mg/kg (1% of the most effective subcutaneous dose) or saline was injected at the L4–L5 level intrathecally using a 10-μl Hamilton syringe (26 gauge, model 801 RN; Hamilton Bonaduz AG, Switzerland) as described elsewhere.\(^1\)\(^8\) Injected volumes were 2 μl for P3, 4 μl for P10, 7 μl for P21, and 10 μl for P40. Animals were then placed back in the testing area for 5 min before further testing. Injection sites were confirmed postmortem.

**Assessments of Levels of Sedation**
The righting reflex was used as a measure of sedation after administration of either midazolam (10 mg/kg) or saline subcutaneously. The baseline righting reflex latency was measured by placing the rat in the supine position and monitoring the time taken to turn over and stand upright using a stopwatch operated by the observer. Three measurements were made at each time point, and the mean latency was calculated. Righting reflex latency was then remeasured 15 min after subcutaneous administration of midazolam or saline. If animals were able to right themselves immediately, an arbitrary value of 0.5 s was awarded. Animals were returned to an upright position if they had failed to do so after 20 s.

**Statistics**
Statistical analyses were performed using GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA). Data from the behavioral studies were found to be nonnormally distributed; therefore, any statistical differences between groups in these studies were determined using nonparametric statistics (Friedman’s test with multiple comparisons followed by a Dunn’s posttest). In physiologic studies, electromyographic responses to increasing intensity of Vfh application were measured and plotted on a graph. The

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*Fig. 1. Subcutaneous administration of midazolam significantly decreased mechanical withdrawal thresholds in P3 rats at all doses tested (A). Conversely, no significant effects of the drug were seen in P10 rats (B). No significant effects of midazolam were observed in older (P21) neonates (C). Comparisons of data from the 15-min postinjection time point reveals significant differences between the responses at P3 and the other three ages (Friedman’s test) (D). Bars indicate mean ± SE. *** \(P < 0.001\), \(n = 6–12\) in each group.*
area under the stimulus response curve at each time point was then determined and normalized as a percentage of the baseline (predrug) area under the stimulus response curve. Data were found to be normally distributed. The effect of drug (or saline) was then determined using parametric statistics (one-way ANOVA with Dunnet’s posttest), while comparisons of responses to drug among age groups were made using Student’s t test.

Results

The Effect of Midazolam on Mechanical Thresholds Differs Significantly with Postnatal Age

The effect of midazolam on the hind paw mechanical withdrawal threshold at three postnatal ages (P3, P10, and P21) was determined using a range of doses (0.1, 1.0, and 10 mg/kg subcutaneously) (fig. 1). The effects differed strikingly with age. At P3, the youngest age tested, midazolam caused a dose-dependent reduction in the mechanical withdrawal threshold not seen with saline (fig. 1a). The effect of midazolam reached a plateau 15 min postinjection at all doses (0.1 mg/kg: $-2.4 \pm 0.7$ Vfh, 1.0 mg/kg: $-3.6 \pm 0.8$ Vfh, 10 mg/kg: $4.6 \pm 0.9$ Vfh) and remained significantly below baseline thresholds for the remainder of the 60-min test period. At P10, midazolam failed to significantly alter mechanical withdrawal threshold at any dose tested ($-0.06 \pm 0.26$ Vfh, $P > 0.05$) (fig. 1b). At P21, midazolam also had no significant effect compared with saline ($-0.56 \pm 0.49$ Vfh, $P > 0.05$) (fig. 1c).

The effect of a 10-mg/kg subcutaneous dose of midazolam on hind paw mechanical thresholds at all three ages and in adults (P40) are compared 15 min postinjection (fig. 1d). Adult rats were refractory to the drug with regard to their mechanical thresholds. Comparisons among the four age groups 15 min postinjection (10 mg/kg) show statistically significant differences in the ability of midazolam to decrease mechanical thresholds for the P3 group compared with all three older ages ($-4.57 \pm 0.86$ Vfh [P3] vs $-0.06 \pm 0.25$ Vfh [P10], $-0.56 \pm 0.5$ Vfh [P21]; $P < 0.001$, n = 8 per group).

The Sedative Effects of Midazolam Are Also Developmentally Regulated

A major property of midazolam and other benzodiazepines is their ability to confer sedation, anxiolysis, and muscle relaxation. We assessed the sedative effects of midazolam in the early postnatal period by measuring the latency for the rats to right themselves from a supine position (righting reflex). In both P10 and P21 rats, the latency to right increased significantly 15 min postinjection (10 mg/kg subcutaneously) (fig. 2). At P10, midazolam increased the latency from 1.4 ± 0.1 to 20 ± 0 s, whereas at P21 the values were 0.5 ± 0 to 19.2 ± 2 s ($P < 0.001$, n = 6 per group). However, at P3, midazolam failed to significantly change the latency to right from $3.2 \pm 0.8$ s (baseline) to $3.9 \pm 0.5$ s ($P > 0.05$, n = 6). It seems therefore that this dose of midazolam has no sedative effect at P3.

Electromyographic Recordings Show That the Age-dependent Sensory Effects of Midazolam Are Not a Function of Sedation

To test the possibility that the postnatal changes in the actions of midazolam on mechanical thresholds at different postnatal ages are a reflection of its sedative action, we measured the effects of midazolam on hind limb flexor electromyographic responses to mechanical stimuli in lightly anesthetized rats at P3, P10, P21, and P40. This technique also allowed us to assess the effects of midazolam over a range of stimulation intensities (from sub- to suprathreshold). Midazolam (10 mg/kg subcutaneously) was administered, and electromyographic measurements were performed both before and 15 min postinjection. The magnitude of electromyographic responses was significantly increased 15 min postmidazolam, compared with baseline values, in the youngest (P3) animals (89% ± 27%; $P < 0.001$, n = 8) (fig. 3a) yet significantly decreased in the oldest (P40) group (87% ± 11%; $P < 0.001$, n = 8). Comparisons among the age groups revealed significant differences between the P3 group and both the P21 and P40 groups ($P < 0.05$ P3 vs P21; $P < 0.001$ P3 vs P40, separate Student’s t tests, n = 6 P21, n = 8 P3 and P40). Figure 3b illustrates the change in the stimulus response profiles for both saline- and midazolam-treated animals in the four age groups. In younger animals, midazolam administration increased the responses to both sub- and suprathreshold stimuli; the area under the curve was significantly greater in the midazolam compared with the saline group. In older animals, however, midazolam decreased respon-
siveness, particularly to suprathreshold stimuli. The area under the curve to midazolam is significantly smaller ($P < 0.05$, one-way ANOVA) in the P40 group. The age-related differences in the action of midazolam were particularly evident when responses to the suprathreshold ($T_0$, $T_1$, $T_2$, $T_3$) stimuli were compared. At P21 and P40, the electromyographic response to suprathreshold stimuli is significantly lower than saline controls (P40: $56 \pm 2.96$ vs $8.58 \pm 1.08$ V, P21: $1517 \pm 634$ vs $2.95 \pm 0.85$ V).

**Assessments of Thermal Nociception Show That the Age-dependent Effects of Midazolam Cross Sensory Modalities**

The effects of midazolam on thermal nociception were also tested using hind limb flexor electromyographic responses evoked by a brief stream of water at 55°C applied to the plantar surface of the hind paw. Figure 4 shows typical examples of the flexor electromyographic trace responses produced by such nox-
ious thermal stimulation at P3 and P21 under light anesthesia. In neonates (P3), midazolam (10 mg/kg subcutaneously) significantly increased the magnitude of the evoked electromyographic response (258% ± 174% of baseline values; \( P < 0.05, n = 4 \)) (fig. 4a) 15 min after drug administration. In contrast, the drug failed to significantly alter the magnitude of the electromyographic response evoked by noxious heating in the P21 group (\( 11\% ± 60\% \text{ of the baseline response} \)) (fig. 4b). Saline produced no significant effects in either age groups (P3: 9% ± 6%, P21: -10% ± 8%; \( P > 0.05 \) in both cases).

**The Age-dependent Sensory Effects of Midazolam Are Not Observed with Spinal Application**

To determine whether the age-dependent differences in the actions of midazolam on mechanical sensory reflexes are mediated at the level of the spinal cord, the drug was administered intrathecally. Figure 5 shows that intrathecal midazolam (0.1 mg/kg) had no significant effect (P3: -0.75 ± 0.56 Vfh, P10: -0.12 ± 0.37 Vfh; \( P > 0.05, n = 6 \) per group) (fig. 5) on mechanical flexor reflex thresholds except in the P21 group, in which thresholds were significantly reduced (\(-0.75 ± 0.17 \text{ Vfh}; P < 0.05, n = 6 \)). This suggests that the age-dependent change in the effects of systemic midazolam are mediated through supraspinal centers rather than at the level of the spinal cord.

**Discussion**

The results show that the effects of the clinically prescribed benzodiazepine, midazolam, on nocicep-

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**Fig. 4.** (A) Histograms illustrating the effects of midazolam on the electromyographic (EMG) response to noxious heat. In neonatal rats, subcutaneous dosing with midazolam significantly increased electromyographic responses 15 min after injection compared with baseline values. No effect was seen in saline-treated animals or in midazolam and saline-treated animals in the P21 group. (B) Typical electromyographic responses in saline and midazolam-treated animals to noxious heating of the plantar foot pad in P3 and P21 rats. Bars indicate mean ± SE. * \( P < 0.05, n = 4 \) per group. Black arrows indicate 55°C water stream application.

**Fig. 5.** The effects of intrathecally administered midazolam (0.1 mg/kg; 15 min postinjection) on mechanical withdrawal thresholds. Thresholds were significantly reduced in the P21 group but in none of the other groups. There were no significant differences among any of the age groups. Bars indicate mean ± SE. *** \( P < 0.01, n = 6 \) per group.
tive processing are strongly dependent on postnatal age. Subcutaneous administration of the drug in neonatal rats had a sensitizing effect, significantly decreasing hind limb flexion withdrawal thresholds and increasing reflex size to noxious mechanical and thermal stimulation of the hind paw. This was in marked contrast to the generally desensitizing effects of midazolam in older and adult rats to suprathreshold stimulation. The sensitizing effect of midazolam was paralleled by an age-related difference in levels of sedation after drug administration. The drug failed to induce any sedation in neonates but significantly sedated older animals. These effects seem to be mediated supraspinally because intrathecal administration of the drug failed to elicit a significant effect on nociceptive activity at any age.

During the 60-min period after subcutaneous administration of midazolam, there was a dose-dependent reduction in mechanical withdrawal thresholds in neonatal, freely behaving rats, yet in older animals there was no effect. Electromyographic recordings in lightly anesthetized animals allowed us to quantify the effects of midazolam over a range of threshold and suprathreshold mechanical stimuli. In the neonate, electromyographic responses were significantly sensitized by midazolam such that stimuli that were subthreshold before drug application became suprathreshold. Furthermore, the magnitude of the reflex evoked by a previously suprathreshold stimuli was significantly increased in the presence of midazolam. In contrast, midazolam decreased responses to suprathreshold stimuli in the adult. Together, the behavioral and electromyographic data show a striking developmental reversal in the effects of midazolam on mechanical sensory processing in the rat.

Using the righting reflex, we also showed that the general sedative effect of midazolam develops between P3 and P10. This raised the possibility that the sensitizing effects of midazolam on mechanical thresholds are masked by sedation at older ages. However, quantitative electromyographic analysis of midazolam’s actions under light anesthesia suggests that the effects on sedation and nociception are on separate developmental trajectories. Whereas the sedative actions are fully established at P10, the desensitizing reflex effects are not yet significant at that age. In fact, the drug is unable to significantly decrease electromyographic responses until P40. The sedative action of midazolam therefore seems separate from the ability of the drug to modulate mechanical reflex thresholds and suggests that midazolam is regulating these phenomena independently. This ability of midazolam to pharmacologically discriminate among behavioral states has been previously observed, with the drug differentially affecting changes in the levels of freezing, exploratory behaviors, and antinociception. Arousal, vigilance, and responsiveness are controlled by nuclei including the dorsal raphe, locus coeruleus, and the posterior hypothalamus. These structures also form part of the supraspinal nociceptive system, and the separation of nociceptive and sedative actions suggests either separate networks or differential pharmacological control of them.

The effects of midazolam were not restricted to mechanical stimuli. The reflex response to high-intensity noxious heating of the hind paw was also increased by midazolam in neonatal rats but not in older animals. This is important as noxious heating exclusively activates high-threshold sensory afferents, providing a purely nociceptive input to the central nervous system, unlike mechanical stimulation, which activates both low- and high-threshold afferents. It is notable that whereas midazolam depressed mechanically evoked reflexes in older animals, it had no effect on heat-evoked reflexes, suggesting that systemic midazolam has no effect on the pure C-fiber evoked activity in normal animals.

Intrathecal application of midazolam allowed us to examine the direct effects of this drug on spinal sensory processing. It has been reported that, in the adult, intrathecal midazolam decreases dorsal horn excitation and increases nociceptive thresholds via a reduction in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor expression. GABA<sub>A</sub> receptors are present both in intrinsic dorsal horn neurones and on primary afferent fibers. We must therefore acknowledge the possibility that the actions of midazolam may be being mediated by a presynaptic action of the drug potentiating inhibition of excitatory sensory input to the spinal cord. In our study, intrathecal application of midazolam failed to evoke any significant changes in nociceptive thresholds except in the P21 group, in which thresholds were decreased. However, most studies showing an ability of midazolam to reduce pain behaviors or to reduce dorsal horn excitation are performed in models of neuropathic or inflammatory pain or in isolated spinal cord slices, whereas intrathecal midazolam has no effect on basal sensory thresholds in sham adult animals, in general agreement with our findings. The differences in effect of midazolam in whole animals and in isolated tissue indicate that the ability of the drug to modulate sensory thresholds is dependent on intact descending inputs from supraspinal sites. This is consistent with our previous finding that in the isolated dorsal horn from both P3 and adult rats, antagonizing the GABA<sub>A</sub> receptor results in disinhibition, yet in intact freely-behaving neonates, antagonizing this receptor results in an increase in sensory thresholds, but a reduction is observed in adults.

It seems that allosteric modulation of GABA<sub>A</sub> receptors by midazolam acts in such a way as to be excitatory in neonatal rats through supraspinal mechanisms. One possible mechanism underlying the developmental changes in GABAergic signaling is the well-established supraspinal shift in Cl<sup>-</sup> reversal potential that has been documented early in the postnatal period. GABA is fundamentally inhibitory in the dorsal horn at P3, where the...
effects of high intracellular Cl\textsuperscript{−} are presumably outweighed by the shunting effect of GABA.\textsuperscript{27,28} However, there is much evidence in higher brain centers that GABA can act as an excitatory neurotransmitter in the neonate, and it is likely that the processes responsible for the differences in action of midazolam occur supraspinally.\textsuperscript{4,29,30} Other factors may have contributed to the differential actions of midazolam in the different age groups. The GAB\textsubscript{A} receptor is a heteropentamer, with benzodiazepine sensitivity being conferred by an interaction between α and γ\textsubscript{2} subunits.\textsuperscript{31–33} Significantly, perhaps there are considerable changes in the subunit composition of the GAB\textsubscript{A} receptor through postnatal development.\textsuperscript{34}

In the clinical setting, midazolam is widely used as a sedative in neonates, but it has been argued that there is insufficient evidence for its use in this population.\textsuperscript{35} Certainly our data support this; however, other drugs that could be used to induce sedation, i.e., chronic opioid infusion, carry significant side effects themselves (for example, opioid-induced hyperalgesia). It is not clear whether midazolam mediates similar effects in the human or at what stage of development this would occur; however, its clinical use has been called into question after a higher incidence of adverse neurologic events in some infants receiving the drug.\textsuperscript{36} Alternative therapeutic strategies are also problematic and carry significant risks themselves. Our lack of understanding of these risks and the way in which drugs act in the neonate requires further study.

References