

## Synergistic interactions between midazolam and alfentanil in isolated neonatal rat spinal cord

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### Summary

Benzodiazepines, which may themselves have analgesic properties, display complex interactions with opioids. This study was designed to investigate the effects of midazolam on nociceptive neurotransmission in isolated neonatal rat spinal cord, and the interactions between midazolam and alfentanil. Slow ventral root potentials (sVRP) were recorded from a lumbar root of spinal cords isolated from 1–7-day-old rats and superfused at 27–28 °C. Midazolam (35 nmol litre<sup>-1</sup> to 15 µmol litre<sup>-1</sup>) significantly ( $P<0.05$ ) depressed sVRP area in a concentration-dependent manner. Midazolam depression was antagonized by flumazenil, bicuculline and naloxone. Midazolam and alfentanil interacted synergistically, as determined by a combination index of less than 1. Midazolam blocked the rebound hyperexcitability observed when alfentanil was reversed by naloxone. The results of the study are relevant to benzodiazepine–opioid analgesia and to the effectiveness of benzodiazepines in mitigating the development of opioid tolerance and dependence. (Br. J. Anaesth. 1996; 77:375–380)

### Key words

Hypnotics benzodiazepine, midazolam. Analgesics opioid, alfentanil. Spinal cord. Rat. Pharmacology, synergism.

A benzodiazepine combined with an opioid analgesic is commonly used for i.v. anaesthesia. There is controversy on whether or not benzodiazepines are analgesics<sup>1–4</sup>; given spinally, their effects appear to be analgesic<sup>1</sup> but systemically their sedative and anxiolytic properties may interfere with the assessment of pain<sup>5</sup>. Complex interactions between benzodiazepines and opioids have been reported with respect to analgesia<sup>2–8</sup>, sedation–hypnosis<sup>8–10</sup> and development of opioid tolerance<sup>11–13</sup>. We have previously reported the effects of µ opioids on nociceptive neurotransmission in isolated neonatal rat spinal cord<sup>14–16</sup>. In response to stimulation of a lumbar dorsal root, a slow ventral root potential (sVRP) lasting 20–40 s can be recorded at the corresponding ipsilateral ventral root. The sVRP is related to nociception by several criteria. The fully developed sVRP requires C-fibre activation<sup>17</sup>. Both NMDA and neurokinin receptors are involved in sVRP generation<sup>18</sup>, and both are linked to pain neurotransmission. An sVRP can be evoked by true noxious stimula-

tion<sup>19,20</sup>. The sVRP is highly sensitive to analgesic agents, including opioids<sup>14</sup> and α<sub>2</sub> adrenoceptor agonists<sup>21</sup>.

The sVRP is depressed by the benzodiazepine diazepam<sup>22</sup>. This study was designed to examine the effects of the benzodiazepine midazolam on the sVRP, and to investigate the interactions between midazolam and the µ opioid alfentanil.

### Materials and methods

After obtaining approval from Stanford's Animal Care and Use Committee, newborn (1–6-day-old) Sprague–Dawley rat pups were anaesthetized with enflurane and decapitated. The spinal cord from the mid-thoracic to sacral level was removed rapidly and perfused at a rate of 4 ml min<sup>-1</sup> with artificial cerebrospinal fluid (ACSF) at 27–28 °C, equilibrated with 95% oxygen–5% carbon dioxide, pH 7.3–7.4. This temperature is physiological for rats of this age when not in close contact with the mother. The ACSF consisted of (mmol litre<sup>-1</sup>): NaCl 123, KCl 5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.3, NaHCO<sub>3</sub> 26, CaCl<sub>2</sub> 2, glucose 30. This concentration of glucose was chosen because it increases viability in a more demanding version of this preparation, the spinal cord with brainstem attached in which spontaneous fictive respiratory discharges are maintained<sup>23</sup>.

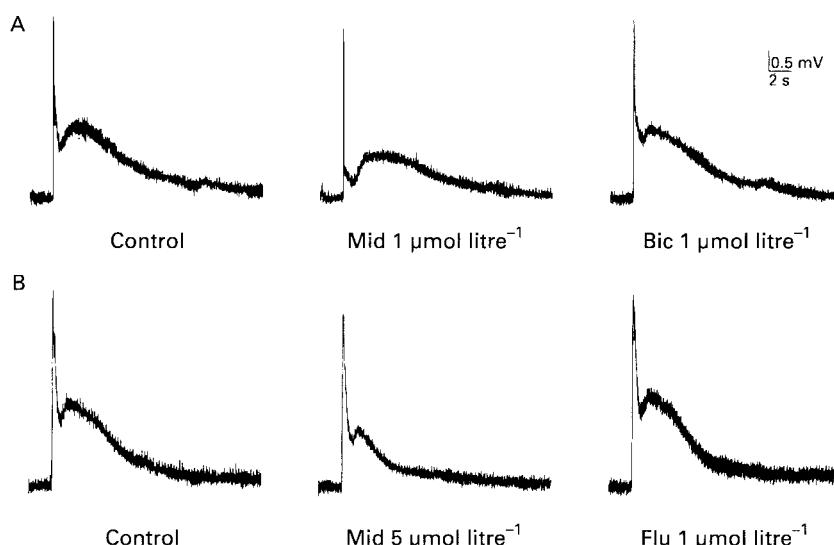
Suction electrodes were arranged to stimulate a lumbar dorsal root and record from the corresponding ipsilateral ventral root. Stimuli were single square wave pulses of 0.2 ms in duration. Stimulus intensity was adjusted to be supramaximal for eliciting the sVRP, and frequency was maintained at 0.02 Hz throughout the study. Responses were digitized, averaged in groups of 5 and stored for later analysis.

Controls for each experiment were the mean of 4–7 averaged responses obtained 30 min before administration of drug. Intermittently throughout the study, time-matched control experiments were carried out. Controls for individual drug experiments and time-matched controls over the duration of a typical experiment were within the range 95–105% of control means. Drugs obtained as pure compound were made up as stock solutions in distilled water,

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**Figure 1** Examples of the effects of midazolam (Mid) on the slow ventral root potential (sVRP). A: Midazolam  $1 \mu\text{mol litre}^{-1}$  depressed the sVRP and the depression was antagonized by the GABA<sub>A</sub> antagonist bicuculline (Bic)  $1 \mu\text{mol litre}^{-1}$ . B: Midazolam  $5 \mu\text{mol litre}^{-1}$  depressed the sVRP and the depression was antagonized by the benzodiazepine antagonist flumazenil (Flu)  $1 \mu\text{mol litre}^{-1}$ .

diluted to the desired concentration in ACSF and applied to the cord in the perfusate for 30–60 min. The pH of the solution of diluted drug was 7.3–7.4. In single-drug studies, each preparation was exposed for 30 min to a single concentration of one agent, then to either drug-free ACSF or an antagonist for 1 h. In combination studies, a fixed concentration of one agent was co-administered with one of a range of concentrations of the other for 30 min; again each preparation was exposed to only one concentration of the second agent.

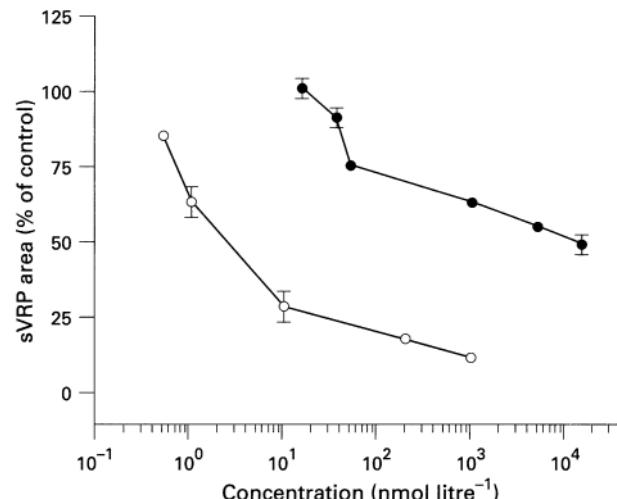
sVRP was quantified as the area under the curve. In a more detailed analysis, the sVRP was divided into an early area, maximally sensitive to NMDA antagonists, from 80 to 440 ms, and a late area, probably mediated via metabotropic receptors of more than one type, from 2.5 to 7.8 s<sup>16</sup>. Results were normalized to control. Slopes of dose-response curves were calculated by linear regression analysis of the log dose-response curve. To test for synergism against additive interactions of midazolam and alfentanil, the combination index (CI) was calculated from median-effect plots for each agent and compared with the predicted value for additivity<sup>24</sup>.

## Results

### MIDAZOLAM DEPRESSION OF SVRP

Midazolam, in concentrations of  $35 \text{ mmol litre}^{-1}$  to  $15 \mu\text{mol litre}^{-1}$ , significantly ( $P < 0.05$  to  $P < 0.0001$ ) depressed sVRP area; examples are shown in figure 1. Depression of the entire sVRP area by midazolam was concentration-dependent (fig. 2). As we have reported previously, alfentanil also depressed the sVRP area more potently than midazolam (fig. 2). Although it appeared that the slope of the midazolam concentration-response curve was shallower than that for alfentanil (midazolam slope  $-15.62$  (SEM 2.375), alfentanil  $-20.92$  (4.622) by linear regression analysis), the difference was not significant.

In previous studies we have shown that both  $\mu$  and  $\kappa$  opioids selectively depressed the slower late phase of the sVRP compared with a relatively fast region sensitive to NMDA receptor antagonists<sup>14,16</sup>. The



**Figure 2** Dose-response curves for alfentanil (○) and midazolam (●) depression of the slow ventral root potential (sVRP). Midazolam exerted significant depressant effects at concentrations of  $35 \text{ nmol litre}^{-1}$  and above. Midazolam was less potent than alfentanil on an equimolar basis and its maximum depressant effect was much less. Data points are mean (SEM) of 4–13 experiments, in each of which the preparation was exposed to a single concentration of one of the agents.

same was not true for midazolam. Midazolam up to  $1 \mu\text{mol litre}^{-1}$  exerted similar effects on the early (80–440 ms) and late (2.5–7.8 s) phases of the response (fig. 3). At higher concentrations, however, an apparently biphasic effect of midazolam on the early phase was revealed; 5 and  $15 \mu\text{mol litre}^{-1}$  continued to exert a concentration-dependent depressant effect on the late phase but appeared to be less effective than lower concentrations in depressing the early phase (fig. 3). This biphasic effect contributed to the shallow dose-response relationship for overall midazolam depression, and to the modest maximum depressant effect.

### MIDAZOLAM-ALFENTANIL INTERACTIONS

Interaction between midazolam and alfentanil was examined by calculating a combination index (CI)<sup>24</sup>. The equation for the CI (eqn (1)) was solved in two ways: for CI using actual concentrations of agents in

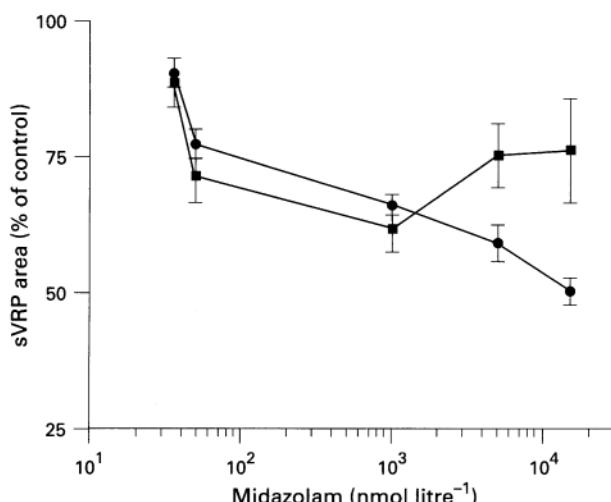


Figure 3 Effects of midazolam on the early area of the slow ventral root potential (sVRP) maximally sensitive to NMDA antagonists, 80–440 ms after the stimulus (NMDA area, ●) compared with effects on the slower metabotropic receptor-mediated component of the sVRP (late area, ○). The effects of midazolam on the two components were similar at concentrations of up to 100 nmol litre⁻¹. Above 100 nmol litre⁻¹, the effectiveness of midazolam on the early area decreased while the later area continued to decline. Data points are mean (SEM) of 5–9 experiments, in each of which the preparation was exposed to a single concentration of midazolam.

combination and the concentration of each agent required to produce a given effect by itself; and for the predicted concentration of one agent required to be added to a known concentration of the other to produce a median effect assuming additivity ( $CI=1$ ). The latter was then plotted on a graph of the actual combination drug effects (fig. 4),

$$CI = CA/A + CM/M + (CM \times CA/MA) \quad (1)$$

where  $CI$ =combination index,  $CA$  and  $CM$ =concentrations of alfentanil and midazolam given in combination to produce a given level of effect, and  $A$  and  $M$ =concentrations of each agent separately required to produce the same level of effect. A  $CI$  value of 1 indicates additivity, less than 1 synergism and greater than 1 antagonism.

When  $CI$  was determined with known concentrations of agents to produce a given effect, values ranged from 0.1 to 0.5, depending on the effect level chosen. This result indicates that interactions between alfentanil and midazolam were synergistic. Synergism was confirmed by the finding that midazolam 15 nmol litre⁻¹, a concentration with no effect on sVRP, significantly potentiated the depressant effect of alfentanil (fig. 4). When  $CI$  was assigned a value of 1 under an assumption of additivity, and the predicted concentration of midazolam or alfentanil required to produce the median effect in combination with the other drug was calculated, the actual curves for midazolam–alfentanil combinations were shifted markedly to the left from the points predicted by the assumption of additivity (fig. 4).

#### RECEPTOR SPECIFICITY OF MIDAZOLAM AND ALFENTANIL ACTIONS

Midazolam is presumed to act at the benzodiazepine receptor site on the  $GABA_A$  receptor. The benzodiazepine antagonist flumazenil and the  $GABA_A$  antagonist bicuculline were examined for their own

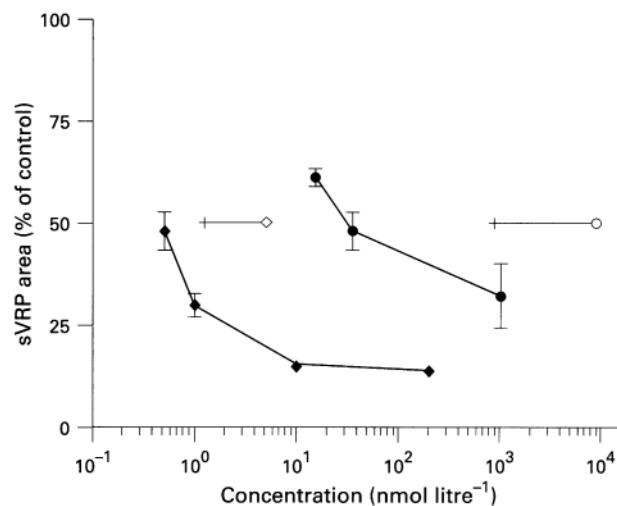


Figure 4 Concentration–effect plots determined when alfentanil was fixed at 0.5 nmol litre⁻¹ and midazolam was varied (●) and when midazolam was fixed at 35 nmol litre⁻¹ and alfentanil was varied (◆). These actual combinations were compared with the predicted median effective concentrations of midazolam in combination with alfentanil 0.5 nmol litre⁻¹ (○) and of alfentanil in combination with midazolam 35 nmol litre⁻¹ (◇), respectively, under an assumption of additivity. Data points from the actual combinations are mean (SEM) of 4–6 experiments, each preparation exposed to one mixture. Predicted concentrations were derived from equation (1) with the value for  $CI$  fixed at 1. Predicted concentrations use median effect plots derived from transformed dose–response curves. Horizontal error bars=95% confidence limits of the regression line drawn through the median effect plots. Both combinations were more effective than predicted by additivity; midazolam and alfentanil interacted synergistically to depress sVRP.

effects and for ability to antagonize the effects of midazolam. Both agents increased sVRP area at concentrations of 500  $\mu$ mol litre⁻¹ and above (fig. 5). Both flumazenil and bicuculline antagonized the effect of low (50 nmol litre⁻¹) concentrations of midazolam at concentrations at which they themselves did not increase sVRP area (50 nmol litre⁻¹); at higher midazolam concentrations (1  $\mu$ mol litre⁻¹), effective antagonism was observed only with higher antagonist concentrations, in the ranges in which the antagonists themselves increased sVRP area (1  $\mu$ mol litre⁻¹).

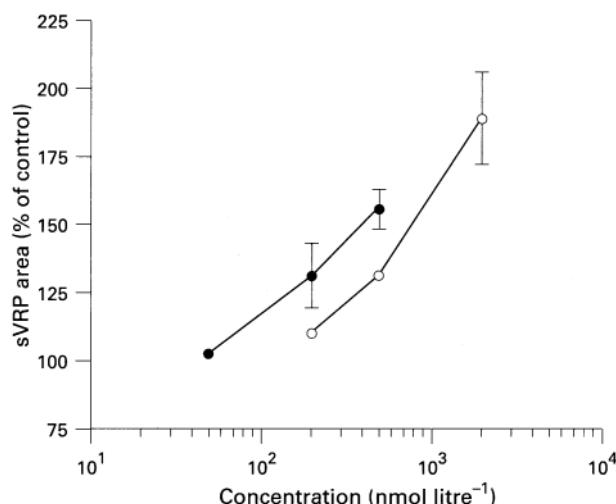
There was unidirectional cross-antagonism between opioid receptor antagonist and the benzodiazepine receptor agonist. The effects of midazolam 1  $\mu$ mol litre⁻¹ were antagonized by the non-specific opioid antagonist naloxone at a concentration that itself had no significant effect on the sVRP (200 nmol litre⁻¹). The  $GABA_A$  antagonist bicuculline (200 nmol litre⁻¹) did not antagonize the effects of alfentanil (200 nmol litre⁻¹).

#### MIDAZOLAM EFFECTS ON OPIOID-INDUCED HYPER-RESPONSIVENESS

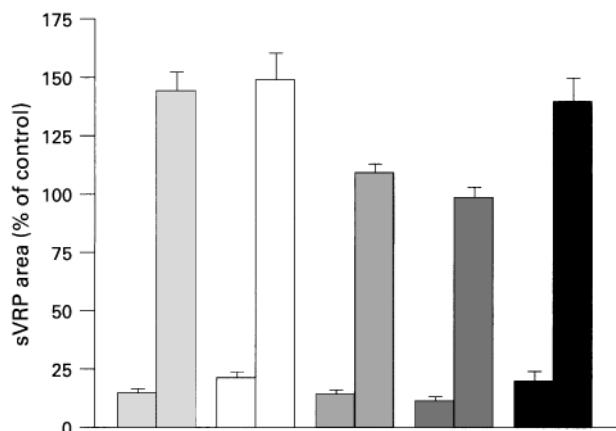
We have observed previously that naloxone, given after  $\mu$  opioids, precipitated an increase in sVRP area to levels above pre-drug controls<sup>14,15</sup>. Midazolam 35 and 1000 nmol litre⁻¹, but not 15 nmol litre⁻¹ blocked this increase (fig. 6). Bicuculline 200 nmol litre⁻¹ did not affect the increase, suggesting that the latter was not mediated via disinhibition at  $GABA_A$  receptors (fig. 6).

#### Discussion

Midazolam itself depresses spinal nociceptive neuro-transmission, as measured by changes in the



**Figure 5** Both flumazenil (●) and bicuculline (○) alone increased sVRP area. The increase was significant ( $P < 0.05$ ) at concentrations of 500 nmol litre $^{-1}$  and above for each agent. Data points are mean (SEM) of 4–6 individual experiments, each preparation exposed to a single concentration of one of the agents.



**Figure 6** Midazolam prevented the rebound hyperresponsiveness observed when alfentanil 200 nmol litre $^{-1}$  was followed by naloxone 200 nmol litre $^{-1}$ . In each pair of bars, the agonist effect is on the left, followed by antagonist on the right. Midazolam 15 nmol litre $^{-1}$  co-administered with alfentanil was not effective, but 35 nmol litre $^{-1}$  and 1 µmol litre $^{-1}$  were. Pale grey = alfentanil followed by naloxone; open bars = alfentanil and midazolam 15 nmol litre $^{-1}$ , with and without naloxone; dark grey = alfentanil and midazolam 35 nmol litre $^{-1}$ , with and without naloxone; medium grey = alfentanil and midazolam 1 µmol litre $^{-1}$ , with and without naloxone; solid bars = alfentanil and bicuculline 200 nmol litre $^{-1}$ , with and without naloxone. Data are mean (SEM) of 5–14 individual experiments.

nociceptive-related slow ventral root potential. The effect, however, is complicated by a biphasic concentration-response curve for the early NMDA receptor-mediated component of the sVRP. The significance of this with respect to analgesia is unclear, although it may be related to a plateau effect and contribute to the very high midazolam doses required for immobility in response to a noxious stimulus (J. Mandema, personal communication). Its implication for midazolam-alfentanil interaction is discussed below.

In an experimental animal model of pain, flumazenil and bicuculline attenuated analgesia produced by subarachnoid administration of midazolam in the lumbar region<sup>1</sup>. Analgesia and a decrease in sVRP area thus result from the action of midazolam on the

benzodiazepine site of a GABA<sub>A</sub> receptor population in spinal cord. Naloxone also antagonized the analgesic effect of both alfentanil and midazolam in animal models<sup>25,26</sup>, indicating that some benzodiazepine effects may be mediated via an opioid pathway in spinal cord. GABA antagonists failed to antagonize opioid effects in animal models<sup>1,25</sup>. The results of our study are consistent with these findings, and suggest that the opioid receptor is downstream from the benzodiazepine receptor in the pathway. Benzodiazepines may act by enhancing release of endogenous opioids or potentiating their effects, or both. In light of marked benzodiazepine potentiation of exogenous opioid agonist effects, the latter possibility seems likely.

The effectiveness of flumazenil and bicuculline as antagonists confirms the reasonable assumption that the effects of midazolam primarily result from actions on the benzodiazepine receptor site on the GABA<sub>A</sub> receptor-ionophore. The increase in sVRP area in the presence of bicuculline alone is not surprising, as there is a marked level of tonic GABA inhibition in the isolated spinal cord, as there is glycine inhibition<sup>27</sup>. The increase observed with flumazenil is surprising. Flumazenil is not known to have inverse agonist effects and is considered to be a pure antagonist at the benzodiazepine site. If this is the case, then the effect of flumazenil itself on the sVRP implies an endogenous ligand at the benzodiazepine receptor.

The synergism observed between midazolam and alfentanil, the ability of naloxone to antagonize the effects of both, and the tendency of midazolam to prevent naloxone-precipitated hyperresponsiveness and the development of opioid tolerance and dependence<sup>12,13</sup> may share a common mechanism, at least in part. We have shown that naloxone-precipitated hyperresponsiveness after  $\mu$  opioids is blocked by the non-competitive NMDA receptor antagonist MK-801<sup>15,16</sup>. MK-801 also potentiated the effect of morphine<sup>16</sup>. To account for these observations, we have proposed that  $\mu$  opioids exert a long-lasting underlying excitatory effect that opposes their overwhelming depressant effect, and that is revealed when the depressant effect is abolished by the antagonist. The excitatory effect is proposed to be related to the initial steps in the development of tolerance or dependence. In common with the latter, it appears to be dependent on NMDA receptors<sup>28–36</sup>. It has been proposed that opioids activate protein kinase C, leading to phosphorylation of the NMDA receptor and increased activity through the latter<sup>37</sup>.

The effect of benzodiazepines on this system may be to decrease activity through NMDA receptors, thus blocking the excitatory effects of alfentanil and apparently potentiating the opposing depressant effects. The mechanism may involve the relative hyperpolarization that occurs when currents are increased at the GABA<sub>A</sub> receptor. Magnesium block of the NMDA receptor is voltage-dependent and enhanced by hyperpolarization. Thus benzodiazepines may block NMDA receptors indirectly and reduce the excitatory effects of alfentanil. In agreement with this hypothesis, propofol, another agent which increases activity at GABA receptors, also potentiates the effect of alfentanil (Feng and

Kendig, unpublished data). Alternative hypotheses are possible, including actions of midazolam on sites other than the GABA<sub>A</sub> receptor and a non-linear summation of the effects of enhanced GABA activity mediated via benzodiazepines and membrane hyperpolarization caused by actions of morphine on a second messenger-modulated potassium channel.

The results of this study are relevant to benzodiazepine–opioid analgesia at the spinal level and to benzodiazepine attenuation of the development of opioid tolerance. Opposing benzodiazepine effects at supraspinal levels may complicate the situation in intact animals when benzodiazepines are administered systemically. Benzodiazepines given intrathecally potentiate opioid analgesia, but given intracerebroventricularly inhibit opioid analgesia<sup>38 39</sup>. GABAergic agonists in periaqueductal grey and rostral ventromedial medulla may inhibit descending inhibition of spinal nociceptive neurotransmission. Benzodiazepines, which enhance GABA<sub>A</sub> actively, may exert opposing spinal and supraspinal effects on nociceptive neurotransmission. Such opposing effects may contribute to the uncertainty regarding their analgesic properties.

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