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Midazolam-induced hyperalgesia in rats: modulation via $GABA_A$ receptors at supraspinal level

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Abstract

The effect of benzodiazepines on the nociceptive threshold was studied in rats using the tail-flick and the formalin tests. Systemic injection of midazolam (10 mg/kg, i.p.) induced a significant decrease of the tail-flick latency and produced a long-lasting nociceptive effect in the formalin test, thus characterising a hyperalgesic state. The hyperalgesia induced by midazolam in the tail-flick test was blocked by flumazenil, a specific antagonist for benzodiazepine sites associated with $GABA_A$ receptors. Picrotoxin, a Cl^- channel blocker, inhibited midazolam-induced hyperalgesia in both tests. Midazolam caused hyperalgesia when administered intracerebroventricularly (i.c.v.; 25 μg) but not intrathecally (i.t.; 75 μg). I.c.v. but not i.t. (5 μg) injection of flumazenil suppressed the hyperalgesia induced by midazolam (10 mg/kg, i.p.). Combination of non-hyperalgesic doses of diazepam (10 mg/kg, i.p.) or ethanol (0.48 g/kg, oral) with midazolam (5 mg/kg, i.p.) also induced hyperalgesia. Our results demonstrate that midazolam and diazepam alone or in combination with ethanol can produce hyperalgesia by interacting with $GABA_A$ receptors at the supraspinal level in rats. The risk of hyperalgesia should be taken in account when these drugs are used in combination in humans.

Keywords: Midazolam; Ethanol; Hyperalgesia; Flumazenil; Picrotoxin; $GABA_A$ receptor

1. Introduction

The effect of benzodiazepines on nociception remains unclear. There have been reports of antinociceptive effects (Yanes et al., 1990; Plummer et al., 1992; Rady and Fujimoto, 1993; Luger et al., 1994), and hyperalgesic effects (Davidovich et al., 1988; Niv et al., 1988; Rattan et al., 1991; Tatsuo et al., 1997) but also of an absence of effects (Moreau and Pieri, 1988; Serrao et al., 1989; Rosland and Hole, 1990; Zambotti et al., 1991; Tejwani et al., 1993) in pain tests. Most, if not all, of the known effects of benzodiazepines are mediated by the interaction of γ-aminobutyric acid (GABA) with $GABA_A$ receptors (Costa and Guidotti, 1979; Tallman and Gallaher, 1985; Matsumoto, 1989). Recently, several subtypes of $GABA_A$ receptors have been identified (MacDonald and Olsen, 1994). The subtypes are characterised by different combinations of the five subunits which constitute the receptor. Sixteen types of subunits have been identified to date, which makes the hypothetical number of $GABA_A$ receptors subtypes very high (MacDonald and Olsen, 1994). It has been shown that benzodiazepine has different affinity for the various $GABA_A$ receptor subtypes (Sieghart, 1995). This interaction is very complex because there is evidence of a hyperalgesic or antinociceptive effect induced by activation of supraspinal $GABA_A$ receptors and an antinociceptive effect induced by activation of receptors located in the spinal cord (Lim et al., 1985; Reyes-Vasques et al., 1986; Baumeister et al., 1988; Drower and Hammond, 1988; Fields et al., 1991; Heinricher and Hilary, 1991). In addition to the route of administration and the type and dose of benzodiazepine used, the pain test used can also contribute to the contradictory results relating to benzodiazepines and nociception. In this study several of these variables were manipulated in order to obtain a better understanding of the complex relationship between benzodiazepine and nociception.

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2. Material and methods

2.1. Animals

Wistar rats weighing 180–250 g (300–400 g for i.t. injections) were housed at a constant temperature of 23 ± 1°C, on a 12-h light–dark cycle, with free access to food and water. The animals which received intracerebroventricular (i.c.v.) or intrathecal (i.t.) catheters were housed individually. The experiments were carried out between 8:00 and 12:00 AM.

2.2. Catheter implantation

Catheters were implanted 7–10 days before the experiments. Animals which showed motor dysfunction after surgery were not used in the experiments.

2.2.1. Intrathecal catheters

Rats undergoing implantation of an i.t. catheter were placed in a stereotaxic frame with the head flexed forward. Under pentobarbital anaesthesia, a 7-cm length of PE-10 tubing was inserted into the subarachnoid space through a slit made in the atlanto-occipital membrane and advanced to the level of the lumbar spinal cord. The external part of the catheter was tunnelled into the skull to exit on the parietal bone. The catheter was fixed by a small piece of acrylic placed between the atlanto-occipital membrane and the skull (Yaksh and Rudy, 1976).

2.2.2. Intracerebroventricular catheters

The rat was placed in a stereotaxic frame following pentobarbital anaesthesia and a hole was bored at coordinates overlaying the left lateral ventricle, i.e., 1.4 mm posterior to the bregma and 1.5 mm left to the midline, according to the atlas of Paxinos and Watson (1986). The guide cannula (a BD-7 stainless steel, 11 mm in length) was inserted 3–3.3 mm into the lateral ventricle and was attached to stainless steel screws placed in the skull.

2.3. Drug administration

When a drug was administered by the intraperitoneal (i.p.) route the volume used was 0.1 ml/100 g animal. I.c.v. and i.t. injections of drugs were done over a period of 90 s to avoid intracranial hypertension or drug extravasation. For i.c.v. injections the volumes used were: 5 μl/animal (midazolam) or 15 μl/animal (flumazenil). For i.t. injections the volume used was 15 μl/animal (midazolam or flumazenil).

2.4. Drugs and vehicles

Flumazenil (Lanexat®, Hoffmann La-Roche) was dissolved in polyoxyethylenesorbitan trioleate (Tween 85, Sigma), 2 drops in 10 ml of distilled water. Picrotoxin (Sigma) and ethanol (Etanol, Grupo Química) were dissolved in physiological saline and midazolam (Dormonid®, Hoffmann La-Roche) and diazepam (Diempax®, Hoffmann La-Roche) were dissolved in the vehicle supplied by the manufacturer. In all tests, control solutions corresponded to the vehicles of the drugs. Formaldehyde (38%, Labsynth) was mixed with saline to obtain 1.25% formalin.

2.5. The tail-flick test

The tail of the animal, 3 cm from the tip, was placed in contact with a spiral wire connected to a heat source. After activation of the heat source (0.6 A, 0.34 cal/min), the number of seconds (the latency) until the rat flicked its tail away from wire was recorded. To prevent tissue damage the cut-off time was 7 s (modified from D'Amour and Smith, 1941). The test consisted of recording a baseline response (usually of 3.5–4.5 s) followed by drug administration and additional measurements at 10-min intervals, from 10 to 60 min after drug injection. The results are presented as the mean of two subsequent recordings. The latency is expressed as the tail-flick index (TFI) obtained by the formula: Lt–Lb/7-Lb, where Lt = test latency, Lb = baseline latency and 7 = cut-off time. All the animals were hand-held during the test phase and were placed back in their home cages during inter-test intervals.

After the experiments the ventricles of the rats with i.c.v. catheters were coloured with Evans Blue (5 or 15 μl). Only the data from rats in which the dye reached the third ventricle and no extravasation occurred were used. This procedure was unnecessary in animals with i.t. catheters because motor impairment usually occurs when the catheter is incorrectly placed.

2.6. The formalin test

Formalin (1.25%) in 50 μl was intraplantarly injected in one of the hindpaws of the animal (time zero), which was then placed in a transparent acrylic box (30 × 20 × 20 cm) for observation. A mirror was placed under the box at an angle of 45 degrees to facilitate observation of the injected paw (modified from Dubbuisson and Dennis, 1977). The degree or severity of nociception is described in terms of the animal's behaviour and how it used the injected paw as follows; degree 0: the paw touches the box wall and floor; degree I: the paw touches the wall and floor lightly and the animal limps; degree II: the paw is not

used, and does not make contact any surface; degree III: the animal licks, shakes or bites the paw. The nociception rate (NR) was obtained from the formula: NR: $(\mathrm{I}t + \mathrm{II}t + \mathrm{III}t/300)$, where t corresponds to the time in seconds spent on each behaviour (degree I, II or III) during a period of 5 min (or 300 s) for each animal. The results are presented either as nociception rate vs. time or as area under curves (AUC).

2.7. Statistical analysis

Data for the time–effect curves were subject to multivariate analysis of variance (MANOVA). In case of interaction between treatment and time, MANOVA was followed by a one-way analysis of variance (ANOVA) for each time. For the analysis of the treatment effect only, MANOVA was followed by ANOVA when AUC was used. For multiple comparisons, ANOVA was followed by Duncan's test. Statistical significance was accepted at $P < 0.05$.

3. Results

No animal showed hyperactivity on handling or signs of intoxication at the doses of agonists and antagonists used.

3.1. Experiment 1—tail-flick test

This experiment involved three treatments: (1) pretreatment of rats with i.p., i.c.v. or i.t. injection of the benzodiazepine midazolam or control solution; (2) pretreatment of rats with i.p. injections of flumazenil (a benzodiazepine receptor antagonist), picrotoxin (a Cl^- channel blocker) or control solution; (2) pretreatment with i.t. or i.c.v. injections of flumazenil or control solution. Pretreatments were carried out 10 min before the test.

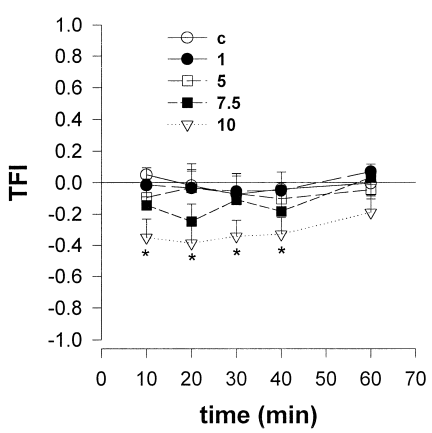

Fig. 1. Dose–response curve (hyperalgesia) for midazolam in the tail-flick test in rats. A significant hyperalgesic effect occurred after injection of 10 mg/kg. The doses of 1.5 and 7.5 mg/kg of midazolam did not change significantly the tail-flick latency. The results are reported as mean values ± S.E.M for tail-flick index (TFI). The symbol (∗) indicates significant difference ($P < 0.05$) from control as determined by the Duncan test for each time point ($n = 8$/group).

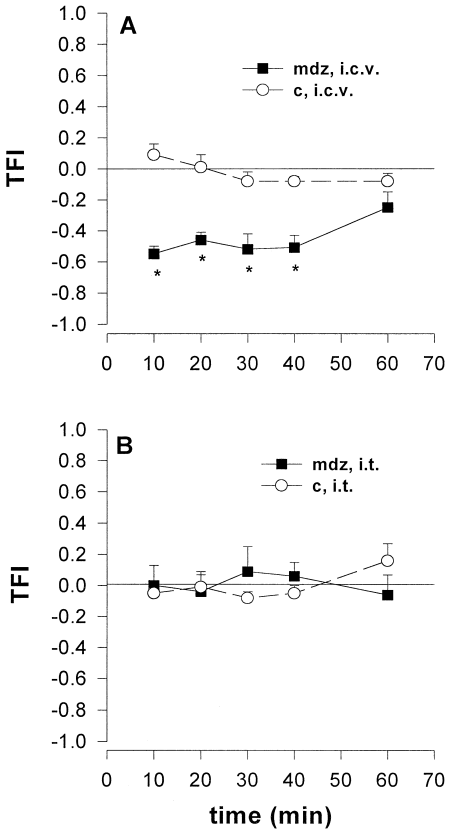

Fig. 2. Effect of central (i.c.v. or i.t.) administration of midazolam in the tail-flick test in rats. In: A) midazolam (25 μg) administered i.c.v. B) midazolam (75 μg) administered i.t. The results are reported as mean values ± S.E.M for tail-flick index (TFI). The symbol (∗) indicates significant difference ($P < 0.05$) from control (animals injected with vehicle of midazolam) as determined by the Duncan test for each time point ($n = 5$/group).

3.1.1. Hyperalgesic effect of midazolam in the tail-flick test

Fig. 1 shows the effect of prior i.p. administration of midazolam in rats in the tail-flick test. The dose of 10 mg/kg induced a significant hyperalgesic effect ($P < 0.05$) but the doses of 1 and 5 mg/kg did not significantly change the tail-flick latency.

3.1.2. Effect of midazolam injected i.c.v. or i.t.

The effect of i.c.v. or i.t. injection of midazolam on the tail-flick latency is shown in Fig. 2A and B, respectively. Midazolam administered 10 min before the test induced a hyperalgesic effect when given i.c.v. (25 μg) $P < 0.05$, but did not induce any effect when given i.t. (75 μg), $P > 0.05$.

3.1.3. Effect of i.p. flumazenil or picrotoxin in the hyperalgesia induced by i.p. midazolam

The effect of flumazenil or picrotoxin on the hyperalgesia induced by midazolam (10 mg/kg, i.p.) is shown in Fig. 3A and B, respectively. Pretreatment with flumazenil (1.5 mg/kg, i.p.) or picrotoxin (0.12 mg/kg, i.p.) significantly inhibited the hyperalgesic effect induced by midazolam (10 mg/kg, i.p.), $P < 0.05$. At these doses, flumazenil and picrotoxin did not produce any significant effect per se.

3.1.4. Effect of intracerebroventricular or intrathecal flumazenil in the hyperalgesia induced by midazolam

The effect of i.c.v. or i.t. injection of flumazenil on the hyperalgesia induced by midazolam (10 mg/kg, i.p.) is shown in Fig. 4A and B, respectively. Flumazenil (5 μg, i.c.v.) completely blocked the hyperalgesic effect of midazolam but did not have an effect per se. I.t. injection of flumazenil (5 μg) neither blocked the hyperalgesic effect of midazolam nor produced an effect per se.

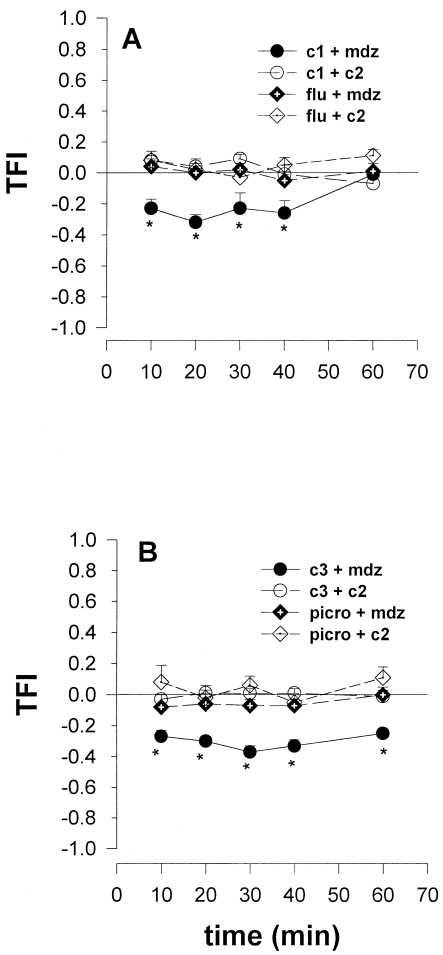

Fig. 3. Effect of pretreatment with flumazenil or picrotoxin on the hyperalgesia induced by midazolam in rats. In: (A) Pretreatment with 1.5 mg/kg of flumazenil (flu). (B) Pretreatment with 0.12 mg/kg of picrotoxin (picro). All animals received two i.p. injections. The pretreatment (vehicle or antagonists) was given 10 min before midazolam (10 mg/kg) or vehicle. The results are reported as mean values ± S.E.M. for tail-flick index (TFI). The symbol (*) indicates a significant difference ($P < 0.05$) from control (animals injected with vehicles), as determined by the Duncan test for each time point. c1 = vehicle of flumazenil, c2 = midazolam and c3 = vehicle of picrotoxin ($n = 8$/group).

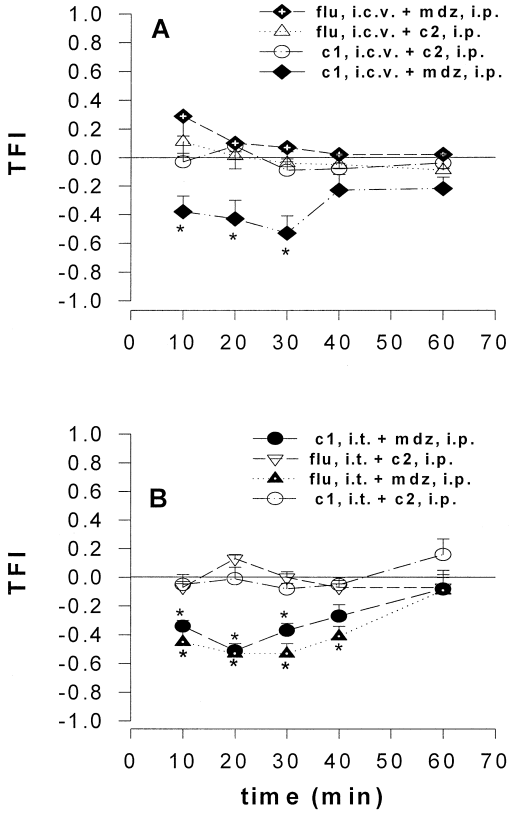

Fig. 4. Effect of intracerebroventricular (A) or intrathecal (B) injection of flumazenil on the hyperalgesia induced by midazolam in the rat tail-flick test. Flumazenil (flu, 5 μg/site) blocked the hyperalgesic effect of midazolam (mdz) only when injected by the i.c.v. route (B). All animals received two injections. The pretreatment (vehicle or flu) was given 10 min before mdz or vehicle (c). The results are reported as mean values ± S.E.M for tail-flick index (TFI). The symbol (*) indicates a significant difference ($P < 0.05$) from control (animals injected with c1 + c2), as determined by the Duncan test for each time point. c1 = vehicle of flumazenil and c2 = vehicle of midazolam ($n = 5$/group).

3.1.5. Effect of ethanol plus diazepam or midazolam in the tail-flick test

Orally administered ethanol in combination with a non-hyperalgesic dose of diazepam or midazolam significantly reduced the tail-flick index compared with the effect of diazepam or midazolam alone, as shown in Fig. 5A and B, respectively ($P < 0.05$). The chosen dose of ethanol did not induce hyperalgesia.

3.2. Experiment 2—formalin test

This experiment involved i.p. injection of midazolam in combination or not with picrotoxin (0.12 mg/kg; i.p.)

before the formalin test. Intraplantar injection of formalin (1.25%) induced a characteristic biphasic nociceptive response, as illustrated in Fig. 6A. This figure shows the nociceptive response (grade I, II and III were achieved) following formalin injection, as reflected by the steep curve in the first 5 min (phase 1). No significant response was observed 10 min after formalin injection, but thereafter there was a long-lasting nociceptive response between 20 and 50 min (phase 2). Previous (10 min) treatment of the animals with midazolam significantly increased the nociceptive response induced by formalin in phase 2 (Fig. 6A, B) $P < 0.05$, thus demonstrating a hyperalgesic component of this response. Higher doses of midazolam (2–10 mg/kg, i.p.) reduced the motor activity of the animals, which interfered with the test. In contrast, 0.12 mg/kg picrotoxin blocked the hyperalgesic effect of midazolam (Fig. 6B), whereas at higher doses it induced an analgesic effect both in the formalin and the tail-flick tests (data not shown). In addition, 0.12 mg/kg picrotoxin did not affect the formalin-induced nociceptive response (Fig. 6B).

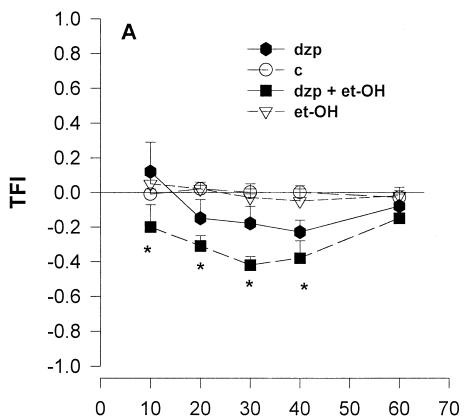

Fig. 5. Potentiation of benzodiazepine-induced hyperalgesia by ethanol in the rat tail-flick test. In: (A) diazepam (5 mg/kg, i.p.) + ethanol (0.48 g/kg, per os). (B) midazolam (5 mg/kg, i.p.) + ethanol (0.4 g/kg, per os). The results are reported as mean values ± S.E.M for tail-flick index (TFI). The symbol (*) indicates a significant difference ($P < 0.05$) from control (animals injected with vehicle), as determined by the Duncan test for each time point. C = control, dzp = diazepam, mdz = midazolam and et-OH = ethanol ($n = 6$/group).

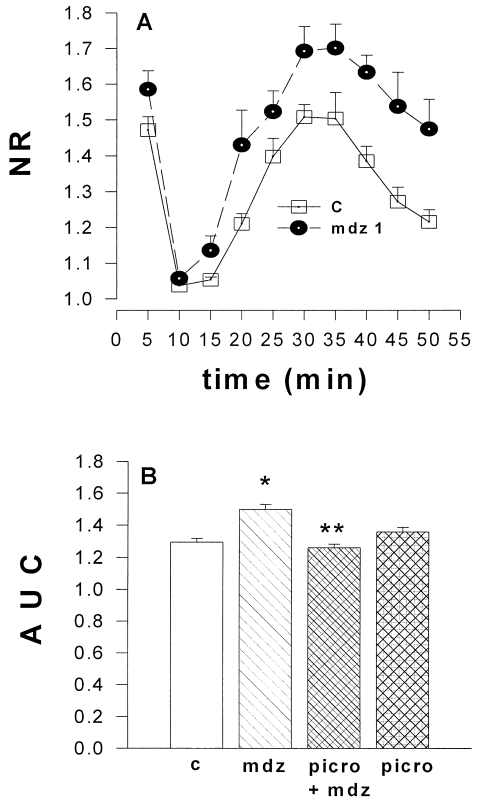

Fig. 6. Midazolam-induced hyperalgesia as detected in the formalin test (A) and its blockade by the chloride channel bloker picrotoxin (B). In A hyperalgesia is described in terms of the nociceptive rate (NR) whereas in B the results are expressed as the area under the curve (AUC) in phase 2. Midazolam (mdz; 1 mg/kg, i.p.) and /or picrotoxin (picro; 0.12 mg/kg, i.p.) were administered 10 and 20 min before intraplantar formalin administration (1.25%), respectively ($n = 6$/group). The symbol (*) indicates significant difference ($P < 0.05$, ANOVA) from control and (**) indicates a significant reduction of midazolam-induced hyperalgesia by picrotoxin ($P < 0.05$, ANOVA).

4. Discussion

Our working hypothesis is that compounds that allosterically interact with the $GABA_A$ receptor, such as the benzodiazepines, induce hyperalgesia (Tatsuo et al., 1997). However, for a long time the effects of benzodiazepines on the nociceptive threshold have remained a matter of apparent controversy. Many authors have claimed that benzodiazepines are hyperalgesic drugs (Davidovich et al., 1988; Niv et al., 1988; Tatsuo et al., 1997), whereas other authors have found them to be analgesic drugs (Yanes et al., 1990; Rattan et al., 1991; Plummer et al., 1992; Rady

and Fujimoto, 1993; Luger et al., 1994), and yet others have reported that they have no effect on the pain threshold (Moreau and Pieri, 1988; Serrao et al., 1989; Rosland and Hole, 1990; Zambotti et al., 1991; Tejwani et al., 1993). However, a careful analysis of the available data has shown that before the nociceptive threshold can be established for a given benzodiazepine it is necessary to establish the doses, route of administration and pain model investigated. For example, studies of the effect of i.p. administration of the benzodiazepine midazolam in the tail-flick test reported a lack of effect in the dose range of 0.03 to 3 mg/kg (Rosland and Hole, 1990; Zambotti et al., 1991; Tejwani et al., 1993), whereas others reported a hyperalgesic effect with a dose of 10 mg/kg (Davidovich et al., 1988; Niv et al., 1988; Tatsuo et al., 1997). These results are not mutually exclusive.

We confirmed in the present paper that only at a dose of 10 mg/kg (systemic) did midazolam induce hyperalgesia, as determined with the tail-flick test in rats. In addition, injection of midazolam i.c.v., but not i.t., reproduced the hyperalgesia induced by systemic midazolam administration. Consistently, either systemic (i.p.) or i.c.v. administration of flumazenil, the specific antagonist of benzodiazepine receptors (MacDonald and Olsen, 1994), or systemic administration (i.p.) of picrotoxin, a Cl^- channel blocker, inhibited the hyperalgesia induced by midazolam. Taken together, these results suggest that: 1. $GABA_A$ receptors are involved in the hyperalgesia of benzodiazepines, and 2. supraspinal rather than spinal levels are involved in $GABA_A$-mediated inhibitory nociceptive neurotransmission.

Studies in the literature evaluating the effects of i.t. injection of midazolam on the tail-flick latency have also given apparently contradictory results. Some reported a lack of effect of midazolam in doses ranging from 16.5 to 50 μg (Moreau and Pieri, 1988; Serrao et al., 1989), whilst others reported slight antinociception over a wider range of doses (10 to 300 μg; Niv et al., 1988; Yanes et al., 1990; Rattan et al., 1991; Plummer et al., 1992; Luger et al., 1994). A possible explanation for these discrepancies may be related to methodological variations. As a whole, our results are consistent with the literature.

Rady and Fujimoto have shown that i.c.v. injections of midazolam (0.25 and 2 μg) inhibit the analgesia induced by i.t. injection of morphine and that this effect is dependent on the release of dynorphin A-(1-17) in the spinal cord. The inhibitory effect was maximal at 0.25 μg midazolam and tended to decrease with higher or lower doses, suggesting a biphasic hyperalgesic effect. These authors also showed that i.c.v. midazolam at 0.5 or 4 μg had no effect, but induced antinociception when given in combination with i.t. injection of dynorphin A-(1-17) receptor antagonists. It is possible that the lack of effect of midazolam in the latter case might be related to a balance between the hyperalgesic effect due to dynorphin A antagonizing enkephalins released in the spinal cord and the analgesic effect mediated by an interaction with supraspinal structures, such as thalamic nuclei, the pre-optic area and substantia nigra. Activation of $GABA_A$ receptors in these structures by agonists is thought to induce antinociception (Lim et al., 1985; Reyes-Vasques et al., 1986; Baumeister et al., 1988). Accordingly, Luger et al. (1994) have reported that midazolam (4 μg, i.c.v.) antagonizes the analgesia induced by i.c.v. morphine but has no effect per se. We concluded that this effect was probably related to the antagonism between GABAergic and opioidergic neurons in structures like the ventral periaqueductal grey matter and the rostro-ventral medulla (Reichling and Basbaum, 1990; William and Beitz, 1990; Heinricher and Hilary, 1991). Taken together, the results suggest that there are several mechanisms that could mediate the supraspinal effects of midazolam on the nociceptive threshold and that the relevance of a particular mechanism is affected by the dose of benzodiazepine given.

Unlike midazolam, diazepam 10 mg/kg i.p. did not induce hyperalgesia in the tail-flick test. Previous studies have reported a lack of effect at doses ranging from 1 to 5 mg/kg (Rosland and Hole, 1990) or slight analgesia (Zambotti et al., 1991). I.c.v. injection of diazepam (1 to 20 μg) induced no effect per se but inhibited morphine induced analgesia (Zambotti et al., 1986; Zambotti et al., 1991). When a combination of diazepam (10 mg/kg, i.p.) and ethanol (0.48 mg/kg, per os) was administered to rats evident hyperalgesia was observed. Since ethanol is reported to be a positive modulator of the binding of benzodiazepine to $GABA_A$ receptors (Davis and Ticku, 1981), we suggest that ethanol facilitates the interaction of diazepam with the $GABA_A$ receptors, uncovering its potential to induce hyperalgesia. In further support of this hypothesis is the observation in the present study that a non-hyperalgesic dose of midazolam (5 mg/kg, i.p.) in combination with 0.48 mg/kg ethanol (per os) also induces hyperalgesia.

We tested midazolam in another model of nociception, the formalin test in rats. The results confirmed our previous findings with midazolam in the tail-flick test. Despite being used in a 10-fold lower dose (1 mg/kg, i.p.) than that used in the latter test, midazolam induced a hyperalgesic effect in the formalin test, and this hyperalgesia was blocked by picrotoxin over the same dose-range used in the tail-flick test. Unlike the tail-flick test, however, the formalin test involves a behaviour besides a reflex (motor) component in its response, and this probably accounts for the differences in the doses required to induce hyperalgesia. In fact, the reduced motor activity induced by doses higher than 1 mg/kg of midazolam affected performance in the formalin test, whereas it did not affect performance in the tail-flick test, so that we could even detect an increased (reflex) response, indicative of hyperalgesia, at 10 mg/kg midazolam in the tail-flick test.

In our opinion, the principal impact of our results is related to the clinical implications of benzodiazepine use.

Benzodiazepine is very often indicated for pre-anaesthetic (parenteral) medication. If this is the case, and because we have shown that the doses that induce hyperalgesia vary depending on the type and route of benzodiazepine administration and on the presence of combined drugs, especially ethanol, the possibility of inducing hyperalgesia should be kept in mind in order to avoid complications in patients at risk.

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