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LABORATORY INVESTIGATIONS

# Antinociception by intrathecal midazolam involves endogenous neurotransmitters acting at spinal cord delta opioid receptors

C. S. GOODCHILD, Z. GUO, A. MUSGREAVE AND J. P. GENT

## Summary

Intrathecal midazolam causes antinociception by combining with spinal cord benzodiazepine receptors. This effect is reversible with doses of naloxone, suggesting involvement of spinal κ or δ but not μ opioid receptors. The antinociceptive effects of intrathecally administered drugs in the spinal cord were demonstrated by measurements of the electrical current threshold for avoidance behaviour in rats with chronically implanted lumbar intrathecal catheters. A comparison was made of suppression by two opioid selective antagonists (nor-binaltorphimine (κ selective) and naltrindole (δ selective)) of spinal antinociception caused by equipotent doses of opioids selective for different receptor subtypes (U-50488H (κ), DSLET and DSBULET (δ), fentanyl (μ)) and the benzodiazepine midazolam. Nor-binaltorphimine selectively suppresed the effects of U-50488H but not midazolam or fentanyl. However, the δ selective antagonist, naltrindole, caused dose-related suppression of antinociception produced by both δ opioid agonists and midazolam with the same $ED_{50}$ (0.5 nmol). We conclude that intrathecal midazolam caused spinally mediated antinociception in rats by a mechanism involving δ opioid receptor activation. (*Br. J. Anaesth*. 1996; **77**: 758–763)

**Key words**

Pain, experimental. Analgesic techniques, regional, intrathecal. Analgesics opioid. Receptors, opioid. Hypnotics benzodiazepines, midazolam. Rat.

Intrathecal injection of midazolam has been shown to produce analgesia in humans[1,2] and antinociceptive effects in rats.[3,4] This effect in rats is caused by an action on $GABA_A$ receptors in the spinal cord and may also be produced by the archetypal benzodiazepine, chlordiazepoxide.[5–7] It has also been shown that the antinociceptive effects of intrathecal midazolam may be suppressed by the opioid antagonist naloxone and this effect was dose-dependent.[5,8] Doses that blocked the midazolam effects were similar to those needed to suppress the effects of the κ opioid agonist ketocyclazocine and significantly greater than those needed to block the effects of the μ selective opioid agonist, fentanyl.[5,8] The results from those experiments implied that intrathecal midazolam, after combining with $GABA_A$ receptors, caused the release of endogenous opioids that acted at a κ or δ but not a μ opioid receptor.

The aim of this study was to investigate the possible role of both of these opioid receptors in the production of spinally mediated antinociception by midazolam. This was achieved by observing the effects on spinal antinociception of different receptor-selective antagonists.

## Materials and methods

These experiments were carried out with the permission of the Licensing Authorities of Great Britain under Home Office Licence No. PPL/50/001310 and in all cases the authors adhered to the Guidelines for Investigation of Pain in Experimental Animals.[9]

### INTRATHECAL CANNULATION

Rats (150–200 g) were anaesthetized with halothane in oxygen-enriched air ($F_{I_{O_2}} = 0.5$) and Portex catheters (id 0.28 mm, od 0.61 mm) were implanted under aseptic surgical conditions into the lumbar subarachnoid space to lie next to the most caudal segments of the spinal cord, as described previously.[10] A minimum period of 18 h elapsed between catheter implantation and nociceptive testing. One experiment was performed on each animal per day up to a permitted maximum of six experiments.

### NOCICEPTIVE TESTS

The electrical current threshold for nociception (ECT) was measured in the skin of the neck and tail every 5 min, as described previously.[10] The standardized response (*r*) was calculated by dividing the three ECT readings obtained 5, 10 and 15 min after intrathecal injection by the three pre-injection readings. In some experiments the tail flick latency (TFL) test was also used. In those experiments TFL

C.S. GOODCHILD*, MA, MB, BCHIR, PHD, FRCA, FANZCA, Z. GUO, MB, BS, PHD (Department of Anaesthesia); A. MUSGREAVE, BSC, J. P. GENT, MA, PHD (Department of Pharmacology); University of Leeds, Leeds. Accepted for publication: August 8, 1996.

*Present address: University Department of Anaesthesia, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria, 3168, Australia. Correspondence to C. S. G.

was always measured 10–15 s before the ECT test was performed, as described previously.[10] The response to the intrathecal drugs was then calculated:

$$\%\,\mathrm{MPE}=\frac{\text{mean TFL post-drug}-\text{mean TFL pre-drug}}{\text{cut-off time}-\text{mean TFL pre-drug}}\times 100$$

AGONIST DOSE–RESPONSE STUDIES

Dose–response relationships for a κ (U-50488H, Upjohn Ltd) and two δ (DSLET, RBI; DSBULET) opioid agonists were investigated. This was done in order to define doses of each agonist which produced just maximal, spinally mediated antinociceptive effects (maximum increase in the tail threshold with no increases in the neck threshold) and which were approximately equipotent with midazolam 46 nmol and fentanyl 0.74 nmol. These doses have been demonstrated previously to be equipotent and produce maximal spinally mediated antinociceptive effects.[10]

The responses ($r$ values calculated as above) were combined for each dose of each agonist to calculate mean (SEM). Dose–response curves were plotted for each agonist and the dose of each agonist which produced a maximal increase in tail ECT with no change in the neck threshold was calculated.

In addition, the time course of the antinociceptive effect was examined for DSLET; electrical current thresholds in four animals who received intrathecal DSLET 9.1 nmol were measured for 40 min after intrathecal injection of drug. The nociceptive thresholds for each test were combined for each testing time between animals and plotted on a time–response curve.

EXPERIMENTS USING NOR-BINALTORPHIMINE

In this group of experiments each rat received intrathecal injection of an equipotent dose of agonist (U-50488H 210 nmol, midazolam 46 nmol or fentanyl 0.74 nmol) given alone at the beginning and again at the end of the series of experiments. Nociceptive thresholds were measured as above. These control responses obtained in the absence of any antagonist were pooled for each drug to derive a mean control agonist response to agonist alone ($R$). In addition, the pre- and post-series control responses for each agonist were compared statistically (Mann–Whitney $U$ test) to test for drug tolerance or cumulative effects of antagonist. In the experiments intervening between the two control agonist responses, a range of doses of nor-binaltorphimine (0.0012–1.24 pmol), dissolved in saline, was injected, mixed with agonist, in the same intrathecal injection.

The responses to nor-binaltorphimine in suppressing the effects of all of these agonists were calculated as percentage suppression of control agonist response from the expression:

$$\%\text{suppression}=\frac{R-r}{R-1}\times 100$$

where $R$ = mean control agonist response in the absence of antagonist and $r$ = response to agonist in the presence of a particular dose of antagonist. The values obtained for each antagonist dose were combined to produce a mean (SEM) and plotted as antagonist dose–response curves.

EXPERIMENTS WITH NALTRINDOLE

In these experiments naltrindole 0.0011–11.1 nmol was given intrathecally dissolved in 5% glucose in a volume of 5 μl mixed with the agonists, and nociceptive thresholds were measured as above. The agonists used were fentanyl 0.74 nmol, U-50488H 210 nmol, DSLET 9.1 nmol, DSBULET 4 nmol and midazolam 46 nmol.

CONTROLS

Five rats with intrathecal catheters were given, on separate occasions, intrathecal naltrindole alone (11.1 nmol) and nor-binaltorphimine (1.24 pmol). The effects of these were assessed with both tests (ECT and TFL). These were the highest doses of antagonists used.

Baseline (pre-drug injection) values for tail nociceptive thresholds were compared with those obtained for the same animal on previous occasions in order to exclude changes induced by progressive neurological damage or residual drug effects. We did not perform control experiments for intrathecal saline alone (the vehicle for some drugs) as we have reported previously that this had no effect on nociceptive thresholds.[3] We did however perform control experiments in four animals who received 5 μl of intrathecal injections of 6% glucose solution which was the vehicle used for other drugs reported in this study.

STATISTICAL ANALYSIS

All data are shown as mean (SEM). Statistical comparisons were made using Mann–Whitney $U$ tests. $P\leqslant 0.05$ was considered statistically significant.

## Results

Evidence that the catheter was intrathecal (a positive lignocaine test) was obtained in all rats after all experiments. We compared the baseline ECT values in the tail for each animal at the beginning of each experiment and there was no change in thresholds over the course of the series which would have suggested neurological damage or residual drug effects. Also, there was no evidence of tolerance to drug effects from comparison of the responses to the same agonists obtained at the beginning and end of the series of experiments with each of the antagonists.

*Table 1* Equipotent doses of U-50488H, DSLET and DSBULET derived from their dose–response relationships

| Drug | Dose (nmol) | ECT (tail) (mean (SEM)) | No. of experiments |
|---|---|---|---|
| U-50488 H | 210 | 1.55 (0.26) | 11 |
| DSLET | 9.1 | 1.97 (0.23) | 6 |
| DSBULET | 3.37 | 1.91 (0.28) | 6 |

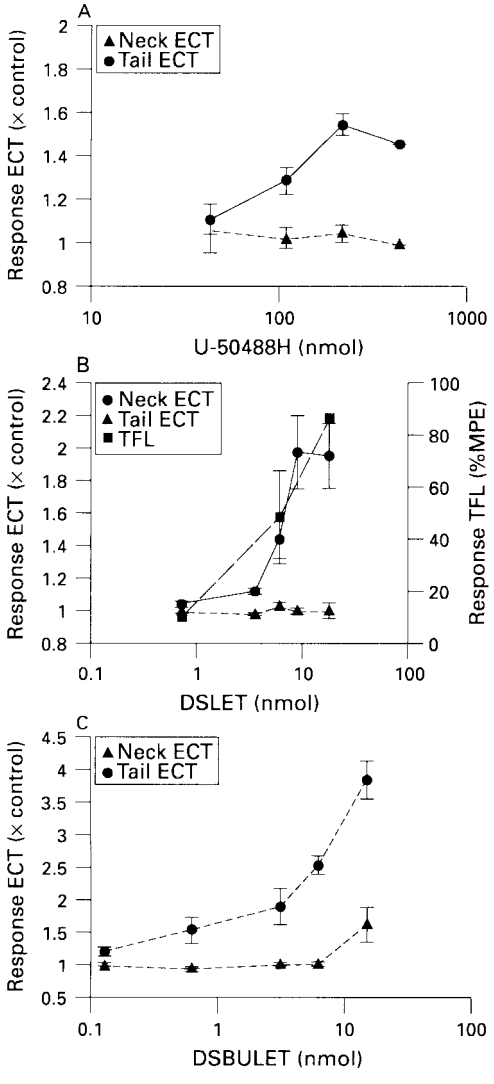


*Figure 1* Dose–response curves for intrathecal U50488H (A), DSLET (B) and DSBULET (C) for neck and tail electrical current threshold (ECT) values and tail flick latency (TFL) (mean, SEM of 22 experiments in 13 rats (A), 31 experiments in six rats (B) and 30 experiments in six rats (C)).

## AGONIST DOSE–RESPONSE STUDIES

All three opioids produced dose-dependent spinally mediated antinociception that was present and had a peak effect within 5 min (fig. 1). The doses of the three agonists, shown by these dose–response studies to produce just maximal segmental antinociceptive effects (increase in tail thresholds with no change in neck thresholds), are shown in table 1.

The antinociceptive effects assessed with the ECT for the δ and κ opioid agonists did not differ significantly from each other or from those obtained in the experiments with antagonists of midazolam (46 nmol; 1.81 (0.1) ECT tail) and fentanyl (0.74 nmol; 1.91 (0.07) ECT tail) (Mann–Whitney *U* test; $P > 0.1$). TFL was also measured in the experiments with DSLET. These measurements were performed only for doses of intrathecal DSLET of 0.728, 6.19 and 18.2 nmol. DSLET caused a dose-related increase in TFL.

Time–response curves were constructed from four experiments in four animals in the group that received DSLET and in six rats that received intrathecal

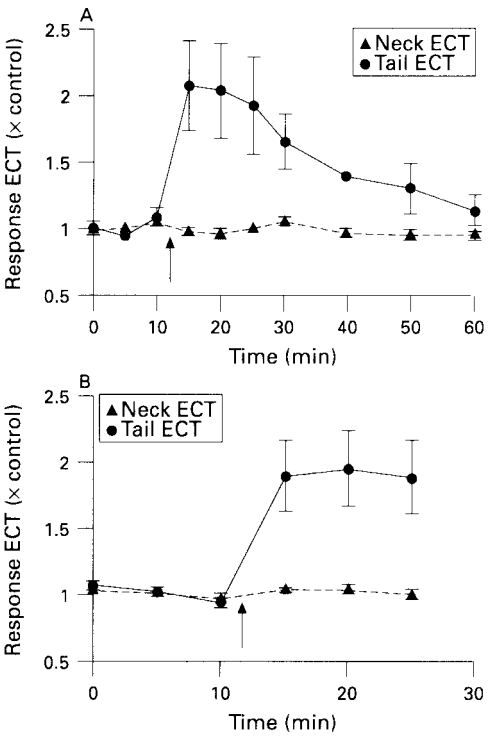


*Figure 2* Time–response relationships for electrical current threshold (ECT) measurements after intrathecal DSLET 9.1 nmol ($n = 4$ rats) (A) and DSBULET 3.37 nmol ($n = 6$ rats) (B) (mean, SEM). Arrows indicate the time of intrathecal injection of drug.

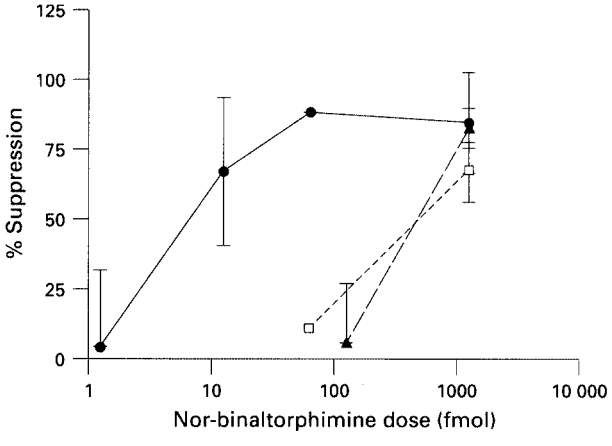


*Figure 3* Nor-binaltorphimine dose–response curves for suppression of ECT antinociceptive effects of intrathecal midazolam 46 nmol (19 experiments, five rats) (▲), fentanyl 0.74 nmol (18 experiments, three rats) (□) and U-50488H 210 nmol (22 experiments, five rats) (●).

DSBULET (fig. 2). Intrathecal DSLET 9.1 nmol produced an increase in the ECT in the tail with no change in the neck and this change occurred at 5 min and persisted for 20 min after injection and regressed towards control values during the following 20 min. Intrathecal DSBULET 3.37 nmol caused a sharp increase in the ECT in the tail with no change in the neck thresholds and this change in the tail was also stable for 15 min after injection.

## EXPERIMENTS WITH NOR-BINALTORPHIMINE

Nor-binaltorphimine dose–response curves for suppression of the antinociceptive effects of U-50488H,

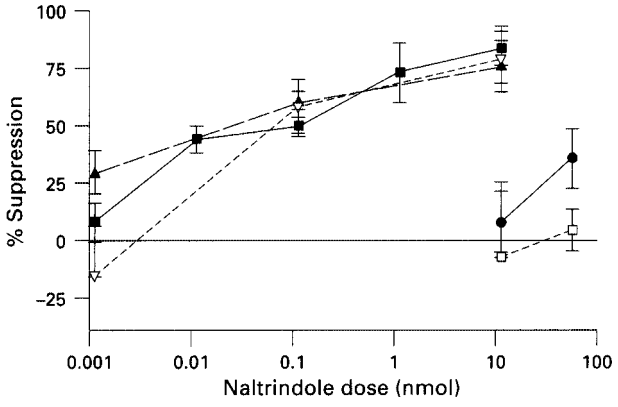


*Figure 4* Naltrindole dose–response curves for suppression of ECT antinociceptive effects of intrathecal midazolam 46 nmol (16 experiments, eight rats) (▲), DSLET 9.1 nmol (24 experiments, eight rats) (■), DSBULET 4 nmol (16 experiments, four rats) (▽), fentanyl 0.74 nmol (eight experiments, eight rats) (□) and U-50488H 210 nmol (eight experiments, eight rats) (●). TFL measurements were also made in DSLET experiments but these are not shown for clarity because the naltrindole dose–response curve for suppressing this effect of DSLET is coincident with the curves for midazolam and the ECT effects of DSLET.

midazolam and fentanyl are shown in figure 3. The selective κ opioid antagonist suppressed only the antinociceptive effects of the κ opioid agonist U-50488H. There was 70% suppression of the effects of fentanyl and midazolam at the highest dose of norbinaltorphimine (i.e. 1.24 pmol); 5% of this dose (0.062 pmol) caused 100% suppression of the effects of U-50488H. Mean control responses before and after the experimental series to agonist alone were, respectively: U-50488H 1.62 and 1.57; midazolam 2.3 and 2.64; and fentanyl 2.14 and 2.08. There were no significant differences between the pre- and post-series controls for each drug (Mann–Whitney *U* test) indicating that no tolerance or cumulative drug effects had occurred.

### EXPERIMENTS WITH NALTRINDOLE

Figure 4 shows naltrindole dose–response curves for suppression of the antinociceptive effects of all agonists tested. Naltrindole, a selective δ opioid antagonist, had no effect on fentanyl or U-50488H spinal antinociception at a dose of 11.1 nmol. In contrast, this dose of naltrindole produced highly significant suppression of spinally mediated antinociception caused by DSLET and DSBULET, the δ opioid agonists, and also midazolam. TFL results for DSLET are not shown in figure 4 in the interest of clarity. However, naltrindole 0.011 and 11.1 nmol caused 41.3 (19)% and 90.26 (10.6) % suppression of the TFL increases caused by intrathecal DSLET 9.1 nmol, the same dose–response relationship as that for suppression of the ECT effects of intrathecal midazolam and DSBULET. The dose–response curves for naltrindole antagonism of both δ opioid agonists and midazolam were coincident. The $ED_{50}$ for this effect, that is the theoretical dose which would cause 50% suppression of spinally mediated antinociception, was the same for all three drugs (approximately naltrindole 0.5 nmol).

### CONTROLS

ECT values in the tail were not changed in the four animals which received 5-μl injections of 6% glucose alone, indicating that this vehicle had no effect on nociceptive thresholds.

The pre and post-series control responses to agonist alone were: DSLET 1.97 (0.23) and 1.9 (0.18) ECT, 64.3 (13.3) and 74.7 (14.6) TFL; DSBULET 2.09 (0.07) and 2.09 (0.29) ECT.

Mean control responses to fentanyl and midazolam given alone were, respectively, 1.91 (0.07) and 1.8 (0.1). There were no significant differences between pre- and post-series control values for each δ opioid agonist given alone and there were no significant differences between control agonist responses, that is they were equipotent in causing spinally mediated antinociception and there was no evidence of tolerance to these agonists in this series of experiments.

## Discussion

In the experiments reported here, we used two important principles to study the receptors responsible for spinally mediated antinociception caused by intrathecally administered agonists. First, in every experiment used for this analysis nociceptive thresholds in the tail increased after drug administration, with no change in neck thresholds. If drug is injected too rapidly intrathecally or at too large a volume, we have found previously that neck thresholds increase also. Second, traditionally, one analyses receptor subtypes using the technique described by Arunlakshana and Schild[11] to calculate the $pA_2$ value. In our experimental preparation the concentrations of agonist and antagonist are unknown and the system does not reach steady state, a requirement for $pA_2$ calculations.

Therefore, the technique of antagonist dose–response curve analysis was used in this as in previous studies.[3 5–7 12] This technique has since been verified by Mackay.[13 14] An antagonist dose–response curve describes the inter-relationship between the antagonist and a native receptor if this receptor causes a physiological effect by binding with exogenous drug or endogenous neurotransmitter. Thus the same antagonist dose–response curve with the same $ED_{50}$ is obtained with a particular antagonist, suppressing the effects of two different drugs, whether they bind to the same receptor, or if one or both causes the release (directly or indirectly) of an endogenous agonist which then binds to the same receptor as the antagonist to produce the effect.

Previous experiments with naloxone indicated that intrathecal midazolam activated a spinal opioid system.[5 8] Benzodiazepine antinociception is probably not a μ opioid effect as fentanyl spinal antinociceptive effects were more sensitive to suppression by naloxone than those of either ketocyclazocine (the archetypal κ opioid agonist) or midazolam[5 8] and naloxone has been shown to display some selectivity for the μ receptor.[15] Although ketocyclazocine is the archetypal κ opioid it is not very selective. However, other studies indicated that a κ opioid ligand with

higher selectivity for the κ opioid receptor than ketocyclazocine, ICI 197067, had a similar $ED_{50}$ for naloxone suppression of its spinally mediated antinociceptive effects.[16] Although naloxone is more selective for the μ opioid receptor, the degree of selectivity is not high and it does not discriminate between δ and κ opioid sites. Clearly further experiments with more selective antagonists are needed. This study demonstrated a very high degree of selectivity for nor-binaltorphimine for antagonism of the effects of the κ opioid agonist U-50488H compared with the μ opioid agonist fentanyl. Spinal antinociception produced by midazolam was not suppressed by the doses of nor-binaltorphimine which were selective for the μ opioid receptor, although some suppression was produced by the higher, non-selective doses of nor-binaltorphimine which also suppressed fentanyl antinociceptive effects.

Naltrindole showed a high degree of selectivity for the δ opioid receptor. Suppression of spinal antinociception caused by the δ selective opioid agonists, DSLET and DSBULET, occurred over a range of doses of naltrindole which were three or four orders of magnitude less than those which caused small amounts of suppression of responses to fentanyl and U-50488H. The naltrindole dose–response curve for suppression of antinociception produced by midazolam was to the left of the curves for the κ and μ opioids and it is coincident with all the curves for suppression of the effects of the selective δ opioid agonists. It may be concluded that intrathecal midazolam produces antinociception by activating a system which involves δ opioid receptors. Furthermore, this system is confined to the spinal cord because all experiments in this study demonstrated a differential effect on tail thresholds, that is tail thresholds increased, with no change in ECT occurring in the neck and thus all drug effects and interactions between agonist and antagonist must have occurred at the spinal cord level. It is clear that the initial step in this process is combination of the drug with a typical $GABA_A$/BZ receptor complex as both bicuculline, a $GABA_A$ antagonist, and flumazenil, a benzodiazepine receptor antagonist, suppressed midazolam spinally mediated antinociception in a dose-related manner.[5,6] However, flumazenil and bicuculline did not block the spinal analgesic effect of μ, κ or δ opioid agonists.[17,18] Therefore, the results indicate that intrathecal midazolam positively modulates the effect of GABA at $GABA_A$ receptors and the effect of this is to cause the release of an endogenous opioid acting at δ opioid receptors. It is not known if these $GABA_A$/BZ receptors are pre- or postsynaptic and on what neuronal elements in the spinal cord they are located. However, the effect of positive modulation of GABA at these receptors is to increase chloride flux into the neurone, thus inhibiting its firing. Thus the effect would be to decrease neurotransmitter release from a presynaptic terminal or postsynaptic cell. We must therefore conclude that the pre- or postsynaptic effect leads to decreased release of an inhibitory neurotransmitter that normally inhibits the neuronal release of an opioid acting at δ opioid receptors.

It has been shown previously that intrathecal midazolam 46 nmol increases ECT values without affecting TFL.[10] There have been reports of TFL increases and mixed antinociceptive and hyperalgesic effects after administration of midazolam.[19] However, studies using the preparation that we used in which tail and neck ECT values were measured have consistently demonstrated increases in tail ECT with no change in TFL or neck ECT in the same experiments.[10] The possibility exists in other studies for spread of drug to the brain and that these supraspinal actions lead to mixed effects and TFL increases.

The dose of midazolam (46 nmol) used in these studies produced spinally mediated antinociception measured by the ECT test and was equipotent with doses of fentanyl and DSLET. However, the δ opioid agonist also caused an increase in TFL and increases in ECT values. This TFL effect must be mediated by a spinal cord mechanism as increases in TFL occurred after intrathecal DSLET at doses which caused increases in ECT in the tail without any change in the neck thresholds. We conclude that these two antinociceptive effects of the δ opioid are mediated by separate spinal cord mechanisms. The first (revealed by the ECT test) may be activated by intrathecal benzodiazepine causing the release of an endogenous δ opioid. The second (revealed by the TFL test) may be activated by exogenous δ opioids or endogenous opioid peptides selective for δ opioid receptors but not those released by midazolam.

These results provide further evidence that intrathecal midazolam produces antinociceptive effects by interactions with spinal systems. There may be useful potentiation of antinociceptive and analgesic effects to be gained by concurrent therapy with a μ selective opioid and midazolam. This suggestion, based on the results presented here, is supported by other studies in rats.[4] These authors reported potentiation of submaximal doses of intrathecal morphine by intrathecal injections of midazolam in the tail flick test. This additive effect is interesting because midazolam alone does not increase TFL, concurring with observations reported previously.[10] The mechanism of this interaction is unknown but the results reported here showed intervening steps involving the release of endogenous neurotransmitter substances. These may be the source of that interaction and potentiation, although more work needs to be performed to verify this suggestion.

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