

The role of opioid receptors in mechanical and thermal pain

Abstract

Some analgesics, including opioids, mediate their action through inhibitory G-protein-coupled receptors (GPCRs). Most opioid agonists act on either the δ -opioid receptor (DOR) or the μ -opioid receptor (MOR) at the spinal level to provide anti-nociception in tests of acute pain. Here, we examine the anti-nociceptive effects of subtype-selective agonists, namely deltorphin II, [D-ALA²,N-Me-Phe⁴,Gly-ol⁵]-Enkephalin (DAMGO); (+)- 4-[(α R)- α [(2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N, N-diethylbenzamide (SNC80) and morphine, on heat or mechanical pain in wild-type (wt) mice to determine the role of MOR and DOR in mediating pain modalities. Our results show no characteristic trend that links analgesia to a pain modality of DOR or MOR subtypes in assays for analgesic efficacy against heat (paw withdrawal and tail flick) and mechanical (von Frey) stimuli. Keywords: Opioid, δ -opioid receptor, μ -opioid receptor, pain modality.

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Introduction

It has been established that opioid receptors can produce anti-nociception, thus inhibiting perception and reaction to painful stimuli.^{1,2} Three opioid receptors, mu (μ), delta (δ) and kappa (κ), have been characterised, each displaying distinctive distribution patterns and pharmacological profiles.^{3,4} The μ and δ opioid receptors (MORs and DORs) are G-protein-coupled receptors (GPCRs) that act through inhibitory transduction systems to inhibit pain transmission upon binding of endogenous opioids (endorphins and enkephalins).⁵ MOR is an attractive drug target, as most opiate analgesics used clinically activate it; however, the various central nervous system (CNS) side effects resulting from MOR activation have directed research in favour of the more selective, safe and efficacious DOR.⁵

A binding specificity exists between particular agonists and receptor subtypes. For example, DOR mediates anti-nociception that can be blocked by δ -selective antagonists but not by μ -selective antagonists.^{6,7} Also, studies using MOR knock-out (MOR-KO) mice have revealed that analgesia mediated by the preferential MOR agonist morphine is attenuated as expected.^{1,2} However, in anti-nociceptive assays, MOR-KO mice also showed decreased anti-nociceptive potency and efficacy in response to the spinally-administered DOR agonists, deltorphin II

and DPDPE.^{1,2,8,9,10} This well-documented phenomenon suggests that either these DOR agonists have a direct action on MORs or that DOR activity is enhanced by the presence of MORs, and that both MORs and DORs are needed to achieve full anti-nociceptive potency by these agonists. This further supports the existence of a functional and/or physical interaction between MORs and DORs, which would require these receptors to be co-expressed.^{11,12}

There is a lack of consensus concerning the anatomical distribution of these receptors, and thus, the mechanism of action of opioid analgesia at the spinal cord level is uncertain. To address this issue, Scherrer *et al.* visualised DOR *in vivo* using a green fluorescent protein reporter (GFP)-tagged DOR knock-in mouse strain, and found DOR to be expressed in a neuronal subpopulation that they suggest does not transduce painful heat signals.¹³ Further supported by a set of behaviour studies, Scherrer *et al.* proposed that MORs and DORs mediate analgesia differentially according to thermal and mechanical modalities, respectively. The results of these experiments, however, are controversial because of inconsistencies with previous literature and concerns about experimental design. Specifically, the design has been criticised because the investigators measured pain in two

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different anatomical locations – thermal pain in the tail and mechanical pain in the paw. Thus, the modality-specific analgesic differences observed may be due to selection of different anatomical locations on the mouse.

The aim of this study is to determine if analgesia mediated by the activation of spinal MORs and DORs is specific for either a thermal or mechanical pain modality. We hypothesise that MOR and DOR agonists do not exclusively inhibit heat or mechanical pain, respectively; rather, we expect them to have different efficacies in each modality. To do so, we will evaluate the anti-nociceptive effects of four intrathecally administered agonists: a non-selective opioid agonist, morphine; a DOR-selective peptide agonist, deltorphin; a more selective, non-peptidergic DOR agonist, SNC80; and, a full MOR-selective and potent peptide agonist, DAMGO. Anti-nociception from these drugs will be measured by responses to thermal and mechanical pain using tail flick, von Frey and paw withdrawal assays.

Materials and methods

Animals

The experimental subjects were 120 male, cluster of differentiation-1 (CD-1) mice (Charles River) that weighed 20-25g. Subjects were housed in age-matched groups of two to five in a temperature- and humidity-controlled environment. They were placed on a 12-hour light/dark cycle to accommodate their nocturnal nature, and had free access to food and water except during testing. Each animal was used up to three times and an interval of about one week was allowed between uses. All experiments were approved by the McGill University Animal Care Committee.

Drugs

Morphine sulphate (Medisca Pharmaceutical), deltorphin II (DELTA) (Tocris) and [D-ALA²,N-Me-Phe⁴,Gly-ol⁵]-Enkephalin (DAMGO) (Tocris) were prepared into serial dilutions in sterile saline and frozen until the day of experiment. (+)- 4-[(α)- α -(2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N, N-diethylbenzamide (SNC80) (Tocris) was dissolved in saline with 0.3% tartaric acid. Drug concentrations ranged from 0.003-20nmol in the paw withdrawal and from 0.3-20nmol in the tail flick and von Frey tests. We obtained full dose-response (D-R) curves for each drug and starting doses were determined from unpublished data from Dr Laura Stone's pain laboratory (McGill University, Montreal, Canada).

Drug delivery

The drugs were injected intrathecally (i.t.) by direct lumbar puncture in awake mice, and were administered by Dr Stone according to the method developed by Hylden and Wilcox.¹⁴ The insertion was considered to be successful when it was followed by a reflexive straightening and lifting of the tail. After proper insertion, the drug was injected in volumes of 5 μ l. The experimenter conducting the tests was blinded to all drugs and doses used.

Anti-nociceptive assay

Tail flick test

Thermal nociception was measured in the tail using the tail flick test.¹⁵ The tail flick test was performed by placing the tail of the mouse in a 52.5°C water bath, a temperature deemed to be safe yet uncomfortable. Animals were gently wrapped in a cloth so that only their tails were exposed.

The bottom two-thirds of the tail was immersed in the water bath, and the latency to withdraw or to flick the tail rapidly was measured. A 12-second cut-off was employed to avoid tissue damage, which corresponds to the maximum possible anti-nociception. Latencies were measured at 10 and 30 minutes post drug administration and the results were conveyed as a percent of the maximum possible effect (% MPE) according to the equation:

$$\% \text{ MPE} = \frac{\text{Experimental latency} - \text{Baseline latency} \times 100}{12 \text{ sec} - \text{Baseline latency}}$$

Radiant heat paw withdrawal assay

Thermal nociception was measured by applying radiant heat to the right hind paw using the paw withdrawal apparatus.¹⁶ Baseline measurements were taken prior to drug administration and were compared to post-drug treatment values. The mice were first allowed to habituate in enclosed plastic cubicles on an elevated glass grid for one hour.

The stimulus was achieved by a moveable radiant heat (infrared, IR=30) source that was directed to the plantar surface of the hind paw, and the latency to withdraw the hind paw was measured. To avoid tissue damage, the cut-off time was set to 14 seconds. Latencies were measured at 10 and 30 minutes post drug administration and the results were conveyed as % MPE according to the equation:

$$\% \text{ MPE} = \frac{\text{Experimental latency} - \text{Baseline latency} \times 100}{14 \text{ sec} - \text{Baseline latency}}$$

Von Frey test

Mechanical nociception was measured in the right hind paw using the von Frey test.¹⁷ The sensitivity to mechanical stimuli was evaluated with calibrated monofilaments (von Frey filaments). The animals were placed on a wire mesh platform and allowed to habituate for approximately one hour. The filaments were then applied, in ascending order, to the plantar surface of the right hind paw to the point of bending, and this was held for three seconds. The results were converted to 50% withdrawal threshold using the GraphPad Prism statistical analysis software. The maximum cut-off force applied was 2g. The 50% withdrawal threshold was measured at 10 and 30 minutes post drug administration.

$$\% \text{ MPE} = \frac{\text{Experimental threshold} - \text{Baseline threshold} \times 100}{2\text{g} - \text{Baseline threshold}}$$

Data analysis

The data from each experiment were averaged and converted to % MPE on Microsoft™ Excel, and were fit to a linear slope using GraphPad Prism. Individual dose and/or time points are expressed as means with standard error of mean. All D-R analyses were performed with the FlashCalc 4.5.3 pharmacological statistics software package. The 50% effective dose (ED₅₀) values were calculated using the linear portion of each curve.

Results

Thermal and mechanical anti-nociception mediated by the MOR-selective agonists DAMGO and morphine, and the DOR-selective agonists SNC80 and DELT were examined using tail flick, paw withdrawal and von Frey tests in wt CD-1 mice.

Time course

To examine the nature of each agonist’s action and the time to the peak of its action, we performed time course analyses of DAMGO, DELT, morphine and SNC80. The time course of morphine is shown (Figure: 1 A-C) in all three tests compared to saline controls. The doses varied from 0.003-20nmol (i.t.) in paw withdrawal and 0.3-20nmol (i.t.), in the von Frey and tail flick assays. The latencies to withdraw increased in a dose-dependent manner (with a few exceptions, tail flick and von Frey), with the 10nmol dose appearing to be more efficacious than the 20nmol one. Higher doses were an exception. In all tests, measurements at 10 minutes were generally more effective than or equally effective as those taken at 30 minutes. In contrast, the tail flick test was the only test where morphine showed a greater anti-nociceptive effect at 30 minutes. Deltorphin and DAMGO’s time courses (Figure 1: D-I) showed similar patterns. In general, animals had increased latencies after 10 minutes compared to saline controls, and the higher dose was the most effective in all three tests. The mice tended to lose anti-nociception after 30 minutes. Deltorphin was almost as effective as DAMGO in paw withdrawal, but its efficacy decreased significantly in the tail flick and von Frey assays. These data suggest that DAMGO can create a higher peak analgesic response than deltorphin, and that the response is reversible, as indicated by a return to baseline after 30 minutes. SNC80 (Figure 1: J-L) did not exhibit analgesic effects in the tail flick test, but it offered a significant latency in paw withdrawal and an above-baseline threshold in the von Frey assay. It had an anti-nociceptive effect at both 10 and 30 minutes and only returned to baseline at 60 minutes.

These data suggest that most of the drugs tested have an analgesic response that increases with time, and that most anti-nociceptive responses are reversible, as indicated by a return to baseline after 30 minutes.

Potency and efficacy

Dose-response curves

To examine the potency and maximal efficacy of each agonist, we generated (D-R) curves for all tests at 10 and at 30 minutes. In the paw withdrawal assay, all drugs produced anti-nociception, with

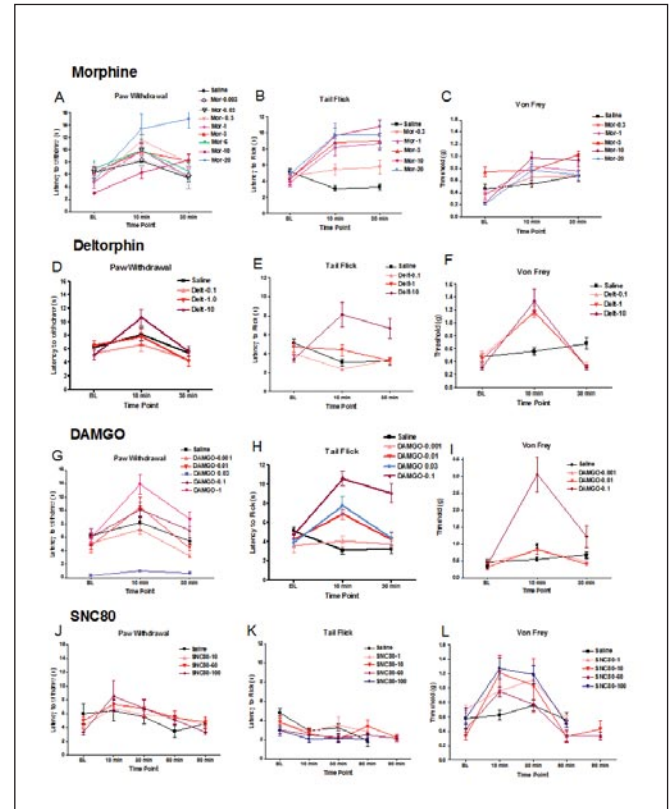


FIGURE 1: Anti-nociceptive time course of morphine (A-C), deltorphin (D-F), DAMGO (G-I) and SNC80 (J-L) in the paw withdrawal, tail flick and von Frey assays.

morphine and DELT as the most potent at 10 minutes. The effect returned to baseline for all drugs except morphine, which was still effective at 30 minutes (Figures 2A and 3A). The tail flick assay was more sensitive to analgesics at both time points than the other tests; anti-nociception increased with dose in all drugs except SNC80, which was comparable to controls (Figures 2B and 3B). The effect was less pronounced at 30 minutes than at 10 minutes with all drugs, with the exception of SNC80 (no change). Significant anti-nociception was seen with all drugs in the von Frey assay, but DAMGO was more potent than the other analgesics at 10 and 30 minutes (Figures 2C and 3C).

Maximum possible effect and 50% effective dose

The dose and % MPE reflects the nature of each agonist’s anti-nociceptive effect and its most effective dose. The ED₅₀ was calculated for each drug and test at the 10-minute time point. In general, the % MPE curves mirrored the responses seen in the D-R curves (Figures 4 and 5: A-C). With the exception of SNC80 in the tail flick test, all drug-treated measurements differed from baseline. Morphine and deltorphin achieved anti-nociceptive efficacy of 80-100% of baseline in the paw withdrawal and tail flick tests. DAMGO was highly efficacious and had reached anti-nociceptive levels of about 170% in the von Frey test.

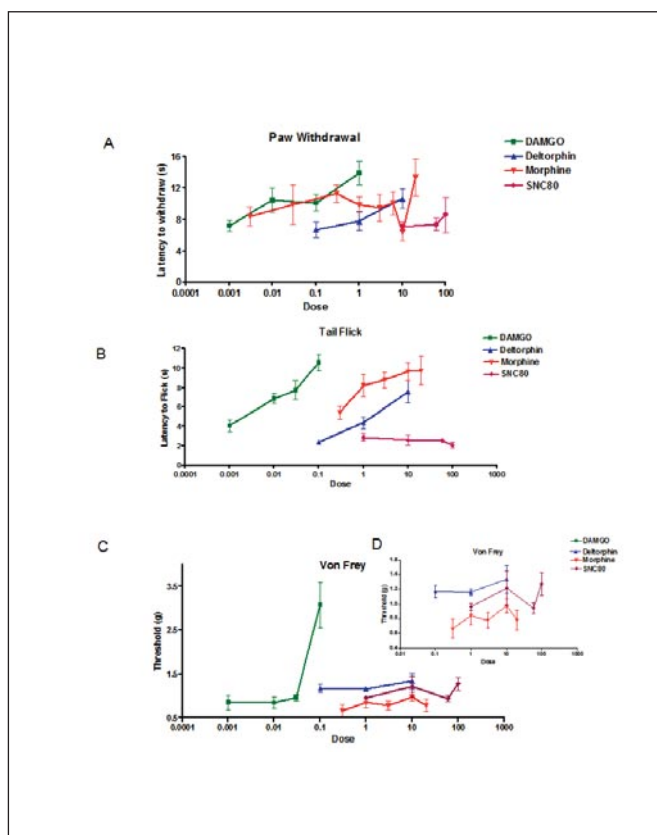


FIGURE 2: The dose-response curves of intrathecally administered drugs DAMGO, deltorphin, morphine and SNC80 in all tests at 10 minutes. (A) Modest anti-nociceptive effect in paw withdrawal in all drugs. (B) All drugs produced anti-nociception in the tail flick assay, except SNC80. (C) In the von Frey assay, some anti-nociception was seen with all drugs. DAMGO was much more efficacious than the others. (D) A magnified image of the von Frey assay that excludes DAMGO shows anti-nociception in the other drugs.

The ED₅₀ values are good indicators of potency, such that a low value that is within the dose range is more potent than a higher value. For morphine, the ED₅₀ values for paw withdrawal and tail flick were 0.0255nmol (CI 0.0023-0.2859) and 0.741nmol (CI 0.0641-8.88), respectively (Table 1). The von Frey test could not be used to generate ED₅₀ values due to the absence of a linear % MPE curve. Thus, morphine was potent in both thermal tests, but values were not calculable in the mechanical test. The ED₅₀ values for deltorphin in the paw withdrawal, tail flick and von Frey tests were 5.05nmol (CI 0.905-28.2), 16.5nmol (CI 6.48-42.1) and 0.997nmol (CI 0.0773-12.9), respectively (Table 1). The ED₅₀ values for DAMGO in the paw withdrawal, tail flick and von Frey tests were 0.0142nmol (CI 0.0018-0.112), 0.0229nmol (CI 0.0121-0.0434) and 0.0079nmol (CI 0.0032-0.0195), respectively (Table 1). In particular, the effect of DAMGO in the von Frey test was the most potent analgesic effect, as reflected by the low ED₅₀. The ED₅₀ value for SNC80 in the von Frey test was 5.78nmol (CI 0.0867-385), while the paw withdrawal and tail flick test data did not show a linear % MPE curve (Table 1).

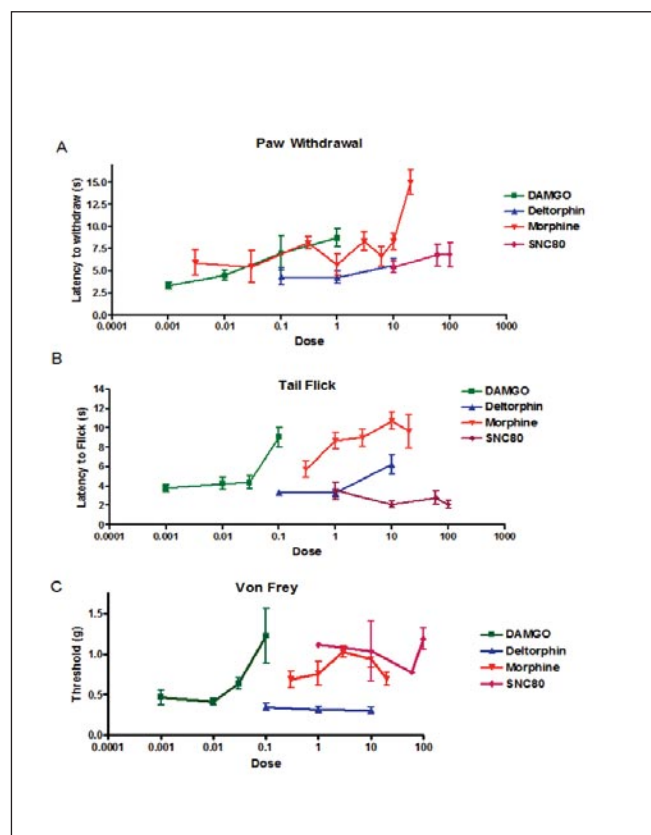


FIGURE 3: The dose-response curves of intrathecally administered drugs DAMGO, deltorphin, morphine and SNC80 in all tests at 30 minutes. (A) A general lack of anti-nociception in paw withdrawal in all drugs except high morphine and DAMGO doses (20 and 1nmol, respectively), which were anti-nociceptive. (B) Anti-nociception in tail flick persisted only in higher doses of morphine and DAMGO. (C) In the von Frey assay, anti-nociception was still present in all drugs except deltorphin.

Efficacy across the three tests

To determine the efficacy of each agonist across the three tests, we plotted % MPE curves at 10 minutes for all drugs in each test (Figure 6: A-D). This allowed us to compare drug-induced anti-nociception in the different pain modalities. While DAMGO and deltorphin produced anti-nociception in all tests, they were both most efficacious in the von Frey test (Figures 6A and 6B). Morphine also produced anti-nociception, but was most effective at low doses in the paw withdrawal test and at high doses in the von Frey test. SNC80 was not efficacious in the tail flick assay and showed limited dose-dependent efficacy in assays applied to the paw. The poor solubility of SNC80 in a physiologically compatible vehicle prevented us from testing higher doses.

Discussion

Modality-specific anti-nociception was measured following intrathecal injections of: the non-selective opioid agonist, morphine; the MOR-selective agonist, DAMGO; the DOR agonist, deltorphin;

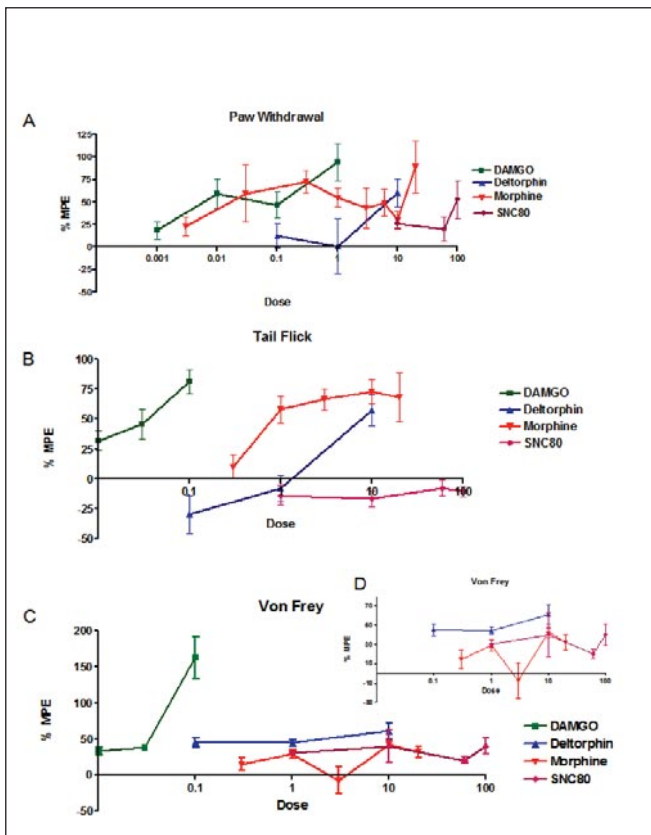


FIGURE 4: The % MPE of intrathecally administered drugs DAMGO, deltorphin, morphine and SNC80 in all tests at 10 minutes showed: (A) A modest dose-dependent anti-nociceptive effect in paw withdrawal in all drugs. (B) Anti-nociception in the tail flick assay was observed in all drugs except SNC80. (C) In the von Frey assay, some anti-nociception was seen with all drugs, except DAMGO, which showed a very effective anti-nociception. (D) A magnified image of the von Frey assay that excludes DAMGO shows anti-nociception in the other drugs.

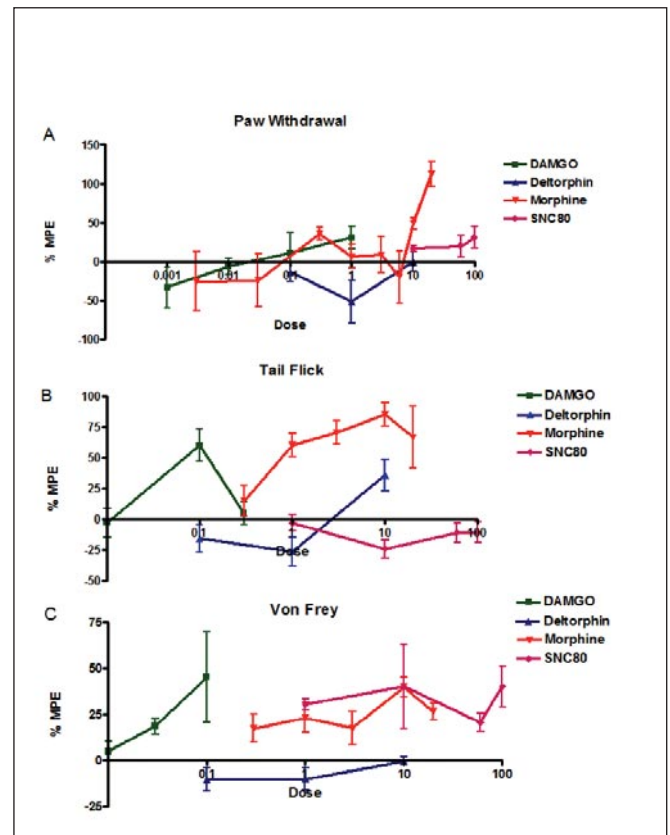


FIGURE 5: The % MPE of intrathecally administered drugs DAMGO, deltorphin, morphine and SNC80 in all tests at 30 minutes. (A) A general lack of anti-nociception in paw withdrawal in all drugs except high morphine doses (10 and 20nmol i.t.), which were anti-nociceptive. (B) Anti-nociception in tail flick persisted in only morphine and DAMGO. (C) In the von Frey assay, some anti-nociception was seen with all drugs, except deltorphin.

Table 1: ED₅₀ values, in nmol, of morphine, deltorphin, DAMGO and SNC80 in paw withdrawal, tail flick and von Frey.

Drug agonist (nmol)	Paw withdrawal (CI)	Tail flick (CI)	Von Frey (CI)
Morphine	0.0255 (0.0023-0.2859)	0.741 (0.0641-8.88)	-
Deltorphin	5.05 (0.905-28.2)	16.5 (6.48-42.1)	0.997 (0.0773-12.9)
DAMGO	0.0142 (0.0018-0.112)	0.0229 (0.0121-0.0434)	0.0079 (0.0032-0.0195)
SNC80	-	-	5.78 (0.0867-385)

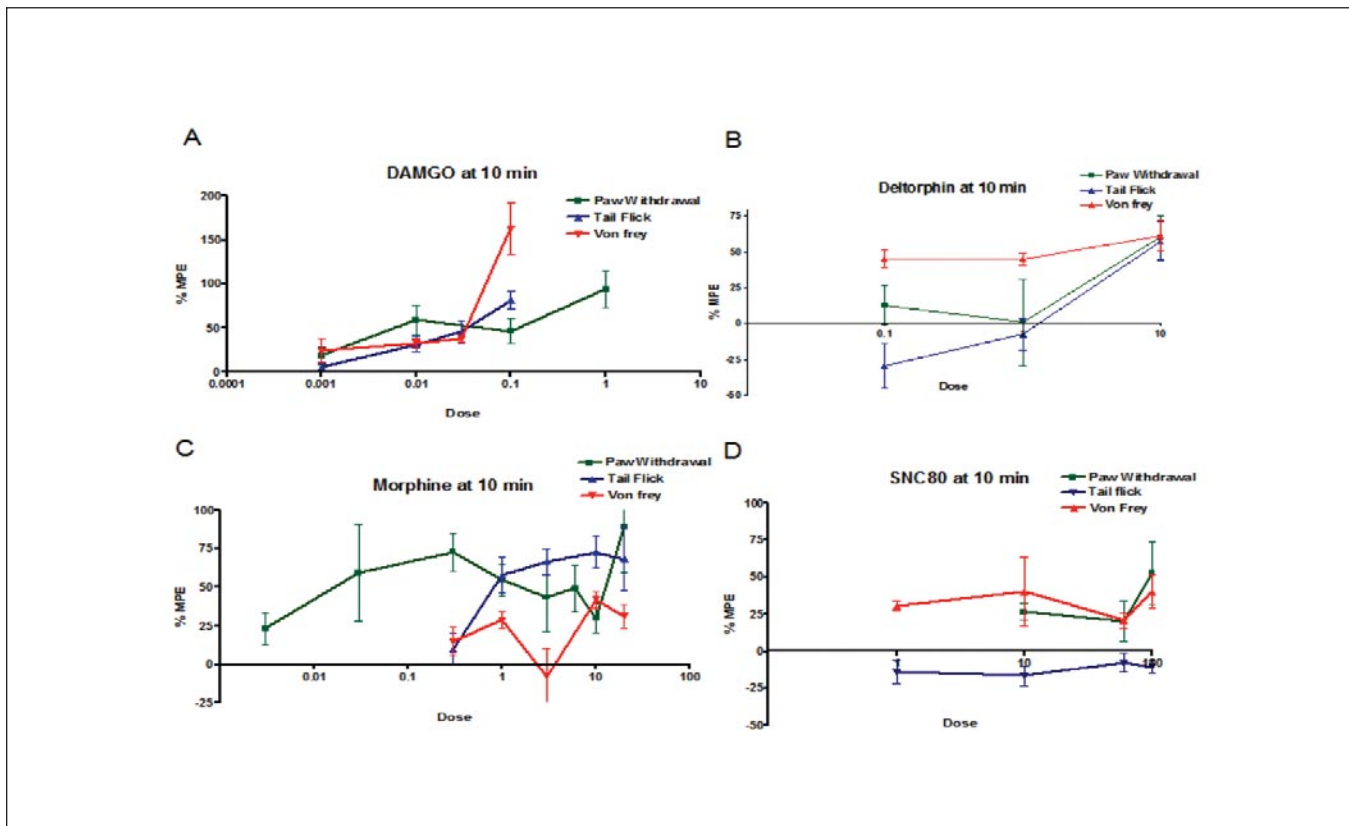


FIGURE 6: Intrathecally administered DAMGO, deltorphin, morphine and SNC80 produce anti-nociception. % MPE curves made to compare the effect of each drug in paw withdrawal, tail flick and von Frey in (A) DAMGO (B) Deltorphin (C) Morphine (D) SNC80. Each data point represents the mean S.E.M.

and, the highly selective DOR agonist, SNC80. Mechanical pain was assessed by the von Frey test and thermal pain was assessed by the tail flick and paw withdrawal tests.

The present study demonstrates that MOR and DOR agonists do not exclusively inhibit heat or mechanical pain. In fact, we show that MOR and DOR agonists have anti-nociceptive effects in both pain modalities. Our results are consistent with previous findings of three lines of MOR-KO mice that retained DOR-mediated anti-nociceptive efficacy from DPDPE and/or deltorphin.^{1,2,8} In fact, Hosohata *et al.* demonstrated that deltorphin, the DOR-selective agonist, produced anti-nociception in the tail flick assay in MOR-KO mice.² Time course and D-R experiments showed no characteristic trend that linked pain modality to opioid receptor subtype. For instance, as shown in **Figure 4**, the MOR agonist DAMGO is the most potent and most effective drug in both thermal and mechanical assays. The less selective DOR agonist, deltorphin, shows moderate anti-nociception in all assays. Additionally, the partial efficacy of the DOR selective agonist SNC80 in both paw withdrawal and von Frey tests does not concur with the subtype-specific analgesic modality theory.¹³ In fact, SNC80 has performed similarly in mechanical and thermal assays; it has equal anti-nociceptive effects in both paw withdrawal and von Frey assays. When the % MPE of the same drug is compared across the three tests, the von Frey assay is almost

always the most responsive to either MOR or DOR agonists at low doses. This challenges the subtype-specific and independent regulation of different pain modalities and instead favours literature that supports the presence of a functional and/or physical interaction between MOR and DOR. Thus, DOR agonists may have a direct action on MORs or enhance anti-nociception at these receptors.^{11,12} Our results support the findings of Guo *et al.* that both MORs and DORs are needed to achieve full anti-nociceptive potency.¹

Although anti-nociception was maintained in MOR-KO mice, there was a decrease in the efficacy and potency of DPDPE and/or deltorphin. This suggests that deltorphin and DPDPE could have off-site targeting on MORs.^{1,2,8} Antagonist studies of MORs with CTOP, a MOR-selective antagonist, showed that CTOP antagonised both DAMGO- and DPDPE-induced antinociception.¹⁸ To overcome uncertainties regarding drug action, mainly receptor specificity, we propose antagonist and MOR/DOR-KO studies that would characterise drug action, since we cannot exclude the possibility that the agonists are acting on the other receptor subtype. In addition, there are limitations in translating these results from the mouse model to human clinical therapies, such as production of more effective drugs that target specific types of pain. Our findings regarding agonist efficacy in both the thermal and

mechanical assays, along with previous MOR-KO studies, do not support Scherrer *et al.* regarding the segregation of pain modalities into opioid subtypes.¹³ However, these results are consistent with their findings that SNC80 does not produce an efficacious anti-nociceptive response in the tail flick assay. We find it to be effective in the paw withdrawal test. This does not conflict with modality used in Scherrer *et al.*'s study; rather, they need to extend the design to include anti-nociception in the paw as well, possibly since the low solubility of SNC80 may prevent it from diffusing to the tail.

Clearly, MOR and DOR agonists have differential efficacies in thermal and mechanical anti-nociception and further research is warranted to elucidate their mode of action within each modality.

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