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ORIGINAL ARTICLE

The effect of intrathecal mu, delta, kappa, and alpha-2 agonists on thermal hyperalgesia induced by mild burn on hind paw in rats

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Abstract
Purpose Mild cutaneous thermal injury, leading to a first-degree burn, induces a sensation of burning pain and enhances the pain sensitivity of the skin. Opioid and α_2 receptor agonists are commonly used to reduce such hyperalgesia. We investigated conditions that induced adequate thermal hyperalgesia in rats and compared the effects of μ, δ, κ, and α_2 receptors at the level of the spinal cord in this model.
Methods A total of 149 male Sprague–Dawley rats were submitted to this study. A first-degree burn injury was induced in the hind paw by contact with a hot plate. The nociceptive threshold was determined by measuring the time from the application of a light beam to the hind paw to the withdrawal response (paw withdrawal latency, PWL). Various hot-plate exposure times and light beam intensities were tested to determine the conditions that induced adequate hyperalgesia. We also tested the effects of intrathecal morphine (μ agonist), DPDPE ([D-Pen2, D-Pen5] enkephalin, a δ agonist), U50488H (trans(+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]-benzacetamide methane sulfonate salt, a κ agonist), and ST-91 (2-[2,6-diethyl-phenylamino]-2-imidazoline, an α_2 agonist) on PWL.
Results A first-degree burn was induced by contact with the hot plate for 45 s. Using current of 5.0 *A*, PWL was reduced by 40% from baseline. Intrathecally administered morphine, DPDPE, and ST-91, but not U50488H, showed dose-dependent antinociceptive effects in both injured and normal paws.
Conclusions Based on these findings, we could find adequate conditions for thermal hyperalgesia model. In this experimental model, μ, δ, and α_2 receptor agonists produced antinociceptive effects at the level of the spinal cord, but the κ receptor agonist did not.

Keywords Mu agonist · Delta agonist · Kappa agonist · Alpha-2 agonist · Thermal hyperalgesia · Intrathecal injection

Introduction

Following peripheral cutaneous injury, the threshold of cutaneous stimulation required to evoke pain sensations is lowered, so that even normally innocuous stimuli may be perceived as painful [1]. A mild cutaneous thermal injury, leading to a first-degree burn (reddened but not blistered or swollen), also induces a sensation of burning pain and enhances the pain sensitivity of the skin. This hyperalgesia results from both direct injury to the skin and various pathologies involving the peripheral and central nervous systems [2]. In central sensitization, the spinal cord is not simply a pathway for pain transmission but is also an important site that induces pathologic pain through the plasticity of painful stimuli.

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Opioid and α_2 receptor agonists are commonly used clinically for pain relief and they are also used to reduce hyperalgesia in animal models. Considerable evidence has shown that μ, δ, and α_2 receptor agonists produce antinociception at the level of the spinal cord [3–7]. However, the effectiveness of κ receptor agonists has been shown to depend on the method of application, including the dose of drug, its method of administration, and the test stimulus [5, 7–9].

In this study, we investigated the conditions appropriate for the induction of thermal hyperalgesia in an animal model. We also tested the association of μ, δ, κ, and α_2 receptors with hyperalgesia at the level of spinal cord in this model by testing the effects of receptor agonists and antagonists.

Materials and methods

Animal preparation

Our study protocol was approved by the Institutional Animal Care Committee of Hanyang University. Male Sprague–Dawley rats (300–350 g; Harlan Industries, Indianapolis, IN, USA) were housed in cages and maintained on a 12-h light–12-h dark cycle. The animals had free access to food and water at all times.

Measurement of the nociceptive threshold

The thermal nociceptive threshold was measured with a device similar to that previously described [10]. A rat was placed in a clear plastic cage (10 cm × 20 cm × 10 cm) above an elevated floor of clear glass (2 mm thick). A radiant heat source (halogen projector lamp CXL/CXP 50 W 8 V; Ushio, Tokyo, Japan) was placed beneath the glass floor. The voltage of the heat source was controlled by a constant current supply. To reduce any variation in the temperature of the plate surface due to room temperature, an under-floor heat source was used such that the temperature of the under-plate was maintained at 30°C. Positioning of the stimulus was aided by an underglass mirror permitting exact visualization of the paw surface to be stimulated.

Following placement in the box and before initiation of the test, the rat was allowed about 30 min to adjust to the environment. The under-floor heat source was positioned to focus on the heel of the plantar surface of the right hind paw. The light was then activated, taking care not to focus on the skin that was off the glass plate. The nociceptive threshold was defined as the time interval, to the nearest 0.1 s, between the application of the light beam and a brisk hind paw withdrawal response (paw withdrawal latency, PWL), which was automatically recorded by a timing circuit. The thermal test system was calibrated prior to each experiment, such that the average PWL in normal untreated rats was 10 ± 1 s. If there was no withdrawal response within 20 s, the rat was regarded as unresponsive and its PWL was recorded as 20 s. Hyperalgesia was defined as a statistically significant decrease in PWL from baseline.

Characterization of thermal injury-induced hyperalgesia

To induce a thermal injury, the rat received isoflurane anesthesia, and the plantar surface of the its right hind paw was placed on a 52.5 ± 1°C hot plate, with a 10-g sand pouch placed on the dorsum of the paw to maintain constant pressure to the heel area during contact. Following the thermal injury, the rat was allowed to recover from anesthesia, with normal spontaneous activity usually observed within 5–10 min.

In the initial phase of the study, we investigated the contact time with the hot plate needed to produce a first-degree burn injury. Rats were divided into 4 groups of 6–7 each, and the baseline PWL was measured in both hind paws. Thermal injury was induced in their right hind paws by exposure to the hot plate for 15 ($n = 7$), 30 ($n = 6$), 45 ($n = 7$), or 60 ($n = 6$) s. PWL was assessed in both hind paws at 15, 30, 45, 60, 90, 120, 150, and 180 min after injury produced with a heat intensity of 5.0 *A*. The site of the injury was monitored to determine whether the thermal injury produced blisters within 24 h. If blistering was noted, the animal was killed.

We also examined the effects of different stimulus intensities on PWL. After baseline PWL was measured in both hind paws, thermal injury was induced in the right hind paw, and PWL in both hind paws was assessed 30, 60, 90, 120, 150, and 180 min later, using heat-evoked stimuli at 5.0 ($n = 6$) and 5.5 ($n = 6$) *A*.

Intrathecal agonists and antagonists

Implantation of chronic intrathecal catheters and preparation of drugs

Chronic intrathecal catheters were implanted according to a modification of a previously described method [11]. Each rat was placed in an anesthesia induction box with isoflurane. After loss of both spontaneous movement and response to a toe pinch, the animal was removed from the box and anesthesia was maintained with a face mask. The surgical area was shaved and blotted with povidone-iodine, the atlanto-occipital membrane was exposed, and a polyethylene-10 catheter was advanced intrathecally through an incision to the level of the lumbar enlargement. The

catheter was externalized on the top of the skull and sealed with a piece of steel wire. The wound was closed with 3-0 silk sutures. Each rat was subsequently housed in an individual stainless steel cage. Rats showing evidence of neurologic deficits, such as gait disturbance, were killed immediately and excluded from the study.

Five to seven days after the surgery, the rats were intrathecally injected with morphine (morphine sulfate; MW = 668.8; Merck, West Point, PA, USA), ST-91 (2-[2,6-diethyl-phenylamino]-2-imidazoline; MW = 253; Böehringer Ingelheim, Ridgefield, CT, USA), DPDPE ([D-Pen2, D-Pen5] enkephalin; MW = 645.8; Bachem California, Torrance, CA, USA), U50488H (trans(+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]-benzacetamide methane sulfonate salt; MW = 465; UpJohn, Kalamazoo, MI, USA), naloxone hydrochloride (MW = 327.37; Du Pont Pharmaceuticals, Garden City, NY, USA), naltrindole (MW = 455.4; a gift from Dr. Michael Rafferty, Searle Research and Development, Skokie, IL, USA), or yohimbine (MW = 354.43; Sigma, St. Louis, MO, USA). Each of these drugs, except for naltrindole, was freshly prepared in physiologic saline, whereas naltrindole was dissolved in 5% 2-hydroxypropyl-β-cyclodextrin. All drugs were prepared such that the required dose was delivered in a volume of 10 μL, followed by flushing with the same volume of saline.

Intrathecal agonist studies

After the measurement of baseline PWL in both hind paws, thermal injury was induced in the right hind paw, with hyperalgesia in the latter confirmed by a reduction in PWL 30 min later. An agonist was immediately injected intrathecally and PWL was assessed 30, 60, 90, 120, and 150 min later in both hind paws. Agonists injected included the μ agonist morphine, in amounts of 0.3 ($n = 6$), 1 ($n = 6$), 3 ($n = 7$), and 10 ($n = 6$) μg; the δ agonist DPDPE, in amounts of 10 ($n = 6$), 30 ($n = 6$), and 100 ($n = 6$) μg; the κ agonist U50488H, in the amount of 100 μg ($n = 6$); the α_2 agonist ST-91, in amounts of 1 ($n = 5$), 3 ($n = 4$) and 10 ($n = 5$) μg; and, as a control, saline ($n = 6$) [3, 4]. Drugs and doses were randomly assigned. We measured behavioral status (agitation, shivering), motor coordination (righting reflex, spinal posture, asymmetric ambulation), and other parameters (pinna reflex, blink reflex) pre- and post-injection of the agonist to evaluate morphine-induced spontaneous agitation and allodynia. There was no rat with abnormal behavior.

Intrathecal antagonist studies

Ten minutes prior to the induction of the thermal injury, rats were intrathecally administered with 30 μg of the opioid antagonist naloxone ($n = 8$), the δ receptor antagonist naltrindole ($n = 8$), or the α_2 receptor antagonist yohimbine ($n = 5$), and PWL values in both normal and injured paws were measured.

We subsequently tested combinations of agonists and antagonists. Combinations included 30 μg naloxone plus 10 μg morphine ($n = 4$) or 100 μg DPDPE ($n = 4$), 30 μg naltrindole plus 10 μg morphine ($n = 4$) or 100 μg DPDPE ($n = 4$), and 30 μg yohimbine plus 10 μg ST-91 ($n = 5$). Drugs and doses were randomly assigned.

To reconcile the peak effect times of agonist and antagonist, antagonist injection time was based on previous studies with naloxone and naltrindole [5, 12] and yohimbine [13], which found that the peak effect time of DPDPE was 15 min and that of morphine and ST-91 was 30 min after intrathecal injection, and that the peak effect times of naloxone, naltrindole, and yohimbine were each 20 min after intrathecal injection. We therefore measured baseline PWL in both hind paws, followed by the induction of thermal injury in each right hind paw. The agonist drugs were injected intrathecally 30 min after the injury. In order to inject the antagonists 20 min before the peak effect of the agonists, DPDPE antagonists were injected 5 min before DPDPE, and morphine and ST-91 antagonists were injected 10 min before their respective agonists. To examine the peak effect of the antagonist for each agonist, we determined PWL 15 min after DPDPE injection and 30 min after morphine and ST-91 injection.

Data analysis and statistics

PWL values were recorded as means ± standard errors (SE). To compare the analgesic effects of drugs, their doses were plotted against their maximum PWLs. The dose–response lines in the injured and normal paws were fitted using least-squares linear regression.

Drug potencies were compared in several ways. We examined the doses required to reverse hyperalgesia in the injured paw and the doses required to produce a maximum effect. As the baseline PWL of each non-injured paw was about 10 s, we determined the effective drug dose required to produce a 10-s PWL in the injured paw (ED10S). We also determined the effective drug dose required to produce a maximum PWL (cutoff time 20 s) for both normal and injured paws (ED20S). All doses were calculated along with their 95% confidence intervals. The slopes of each regression line and the 95% confidence intervals were also calculated. Dose–response curves and statistics were analyzed using the software programs of Tallarida and Murray [14].

Differences in PWL at each testing period, and differences between baseline values and values at each time were compared by repeated-measures analysis of variance

(ANOVA), with subsequent comparisons made using Fisher's protected least significant difference test for multiple treatments. Other comparisons between groups were performed using one-way ANOVA and Dunnett's test. A P value of <0.05 was considered statistically significant.

Results

Thermal injury-induced hyperalgesia

In the absence of thermal injury, baseline PWL was 9.92 ± 0.64 s. Although thermal exposure times of 15 and 30 s had no effect on PWL throughout the entire experimental period, exposures for 45 and 60 s resulted in significant decreases in PWL from baseline, peaking 30 min after the injury (Fig. 1). The magnitudes of the reductions in PWL were 6.28 ± 1.79 s for 45-s exposures and 6.44 ± 0.93 s for 60-s exposures. These reductions in PWL were maintained for 90 and 120 min after exposures for 45 and 60 s, respectively.

Evident blisters on the injured surface 24 h after the thermal injury were observed in 1 of 7 rats exposed to heat for 45 s and in 3 of 6 rats exposed to heat for 60 s. However, no blisters were observed in any rat exposed to heat for 15 or 30 s. Following exposure for 45 s, we usually observed redness of the tissue without blisters. This redness resolved without permanent tissue damage within 24 h.

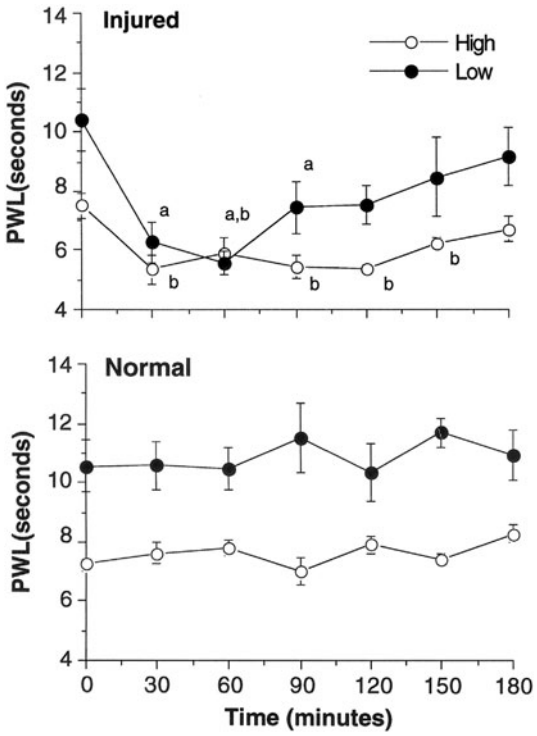

Fig. 2 Time courses of PWL changes measured with high- and low-intensity stimuli focused on the ventral surfaces of both the injured and normal hind paws. *PWL* Paw withdrawal latency. [a]$P < 0.05$ compared with baseline PWL at time 0 using low-intensity stimuli, [b]$P < 0.05$ compared to baseline PWL at time 0 using high-intensity stimuli

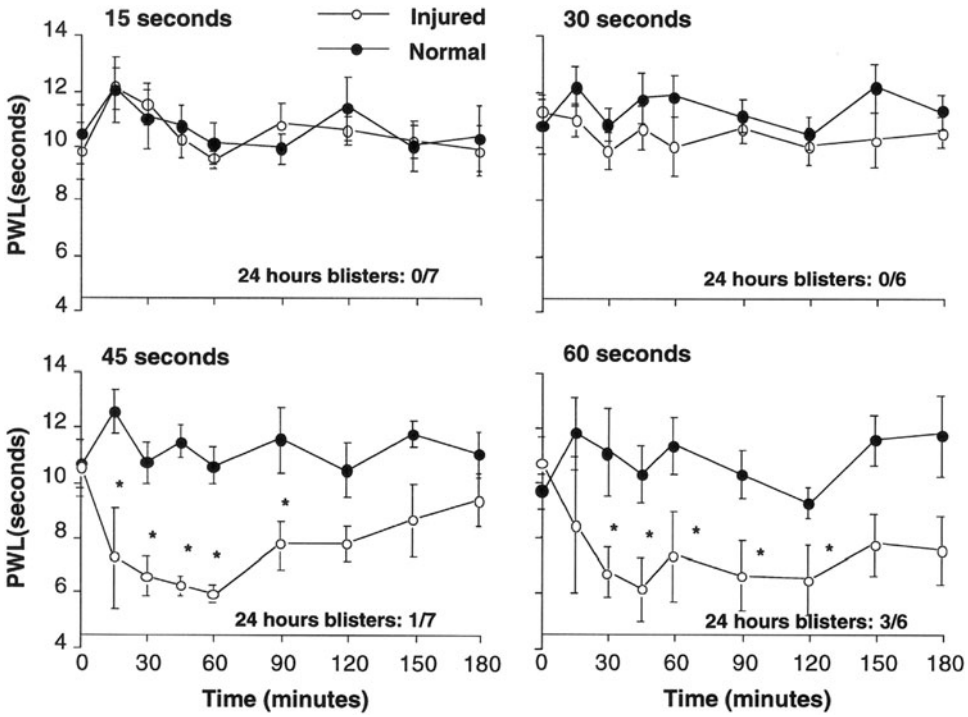

Fig. 1 Time courses of PWL changes in both injured and normal hind paws. Burn injury was induced by placing the right hind paw of each rat on the surface of a hot plate for 15, 30, 45, and 60 s. The PWL of the injured paws decreased after exposure to heat for 45 and 60 s, lasting for up to 90 and 120 min, respectively. In contrast, the PWL of normal left hind paws was not changed. *PWL* Paw withdrawal latency. *$P < 0.05$ compared with baseline PWL at time 0

Figure 2 shows the PWLs on both paws over time in rats exposed for 45 s to heat at high (5.5 *A*) and low (5.0 *A*) intensity. Using 5.0- and 5.5-*A* currents, we observed 40 and 29% reductions in PWL, respectively, at 30 min. Based on these results, we chose as conditions for subsequent intrathecal drug studies contact with the hot plate for 45 s and a heat stimulus of 5.0 *A*.

Intrathecal agonist studies

Intrathecally administered morphine, DPDPE, and ST-91, but not U50488H, showed antinociceptive effects. Following the intrathecal injection of morphine, DPDPE, or ST-91, we observed significant PWL prolongation, with the maximum antinociceptive effects in both injured and normal paws occurring 30 min after the injection (Fig. 3).

The antinociceptive effects of morphine, DPDPE, and ST-91 were also found to be dose-dependent (Fig. 4). From the calculated ED10S and ED20S values, we found that the order of drug potency in this hyperalgesia model was morphine > ST-91 > DPDPE > U50488H (Table 1).

Intrathecal antagonist studies

Table 2 shows the effect of each antagonist alone administered intrathecally 10 min prior to the thermal injury. None of these antagonists showed a significant effect compared with the control group.

When we combined an agonist and an antagonist, we found that naloxone suppressed the effects of intrathecal morphine and DPDPE; naltrindole blocked the effects of DPDPE, but not morphine; and yohimbine reversed the effects of ST-91 (Table 3).

Discussion

A concomitant feature of burn injury is the associated pain that is almost invariably present at all times and is exacerbated during procedures such as dressing changes [15]. Clinical management of burn injury-induced pain is challenging, especially because of the limited knowledge of the basic mechanisms causing these altered pain responses.

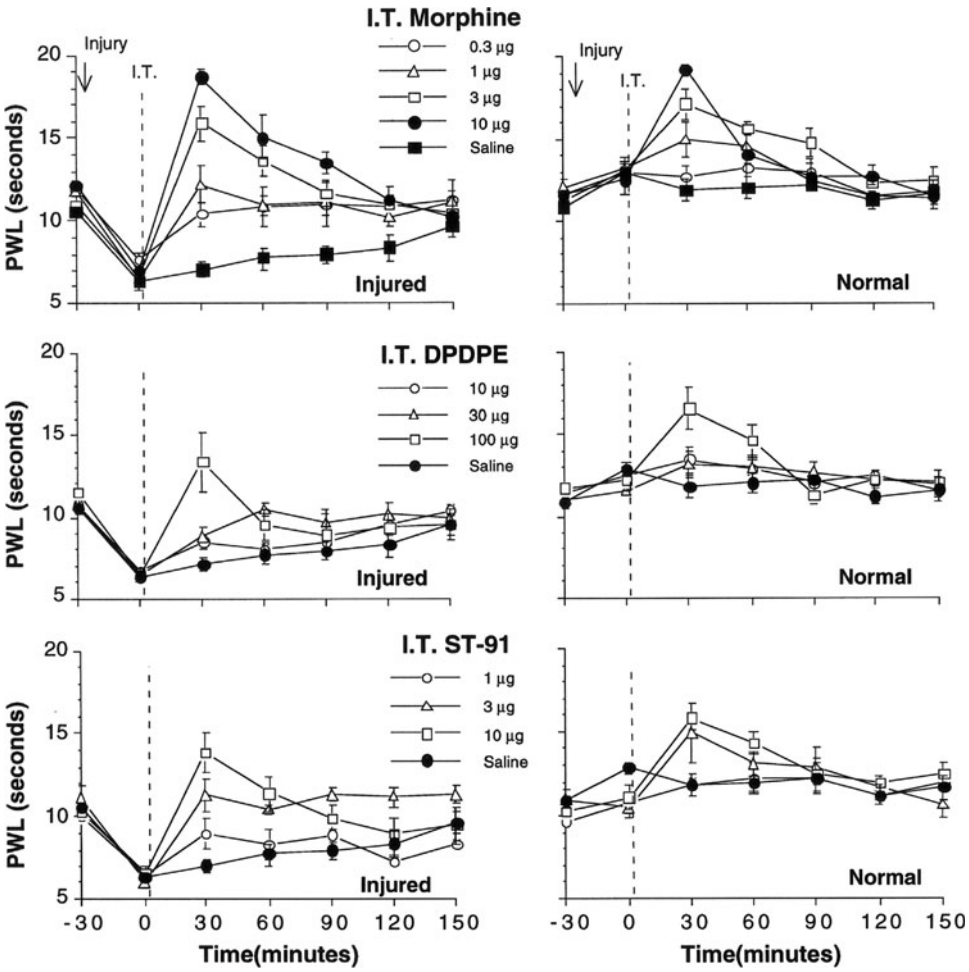

Fig. 3 Time courses of PWL in both the injured and normal hind paws following intrathecal (*I.T.*) injections of agonists. Morphine, DPDPE, and ST-91, but not U50488H, showed antinociceptive effects. Baseline PWL was determined and mild burn injury was induced at time −30 min. Post-injury PWL was measured and the agonist drug was injected intrathecally at time 0. *PWL* Paw withdrawal latency

Burn pain occurs following processes of physical tissue destruction, caused by scalding, flames, or electricity, as well as by chemical agents and radiation contact. There are thermal injury models in humans [16, 17] and rodents [18, 19] have been used to determine behavioral changes, as well as hyperalgesia. Although a recent rat model showed hind paw hyperalgesia after burn injury to the flank area [19], it is not practical to use this model to assess the development of nociceptive behaviors around the area of a burn injury. In addition, little is known regarding the relationship between the severity of a burn injury (i.e., first-, second-, or third-degree) and the development of nociceptive behaviors. While several factors (e.g., the surface area, degree, and site of burn injury) contribute to pain following a burn injury, the degree of the burn injury is certainly an important contributory factor [20]. More importantly, the treatment of pain caused by a burn injury (e.g., with opioids) is likely to be influenced by the severity of the injury [20].

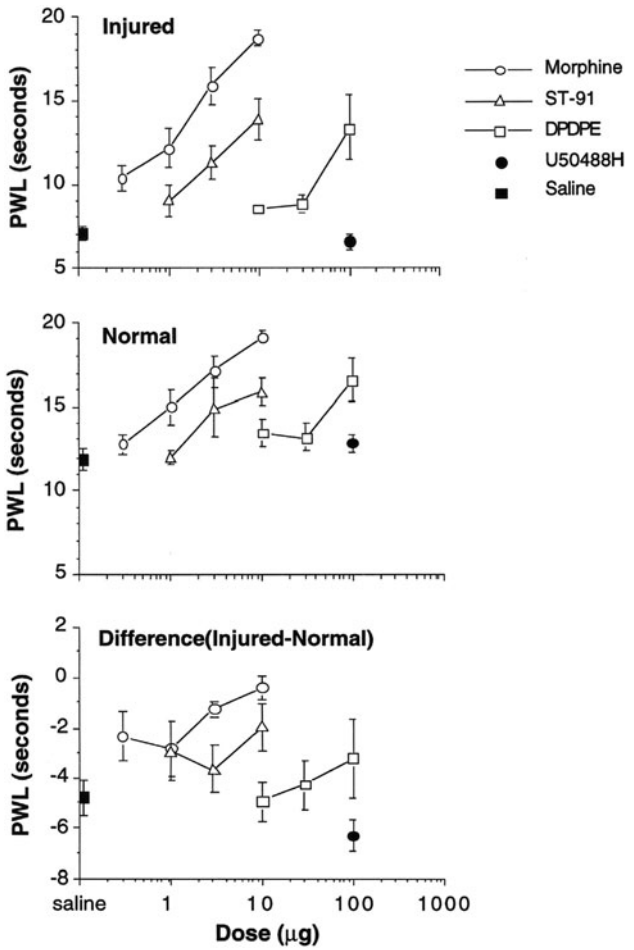

Fig. 4 Dose-response curves of the effects of intrathecally administered morphine, ST-91, DPDPE, and U50488H on mild burn-induced hyperalgesia. *PWL* Paw withdrawal latency

In attempting to determine an adequate heat exposure time in the present study, we found that thermal hyperalgesia in the right hind paws occurred following exposure for 45 s and lasted for at least 1.5 h after the initial burn injury. This duration of exposure produced mild cutaneous injury, without blisters or with few blisters, which resolved within 24 h. During the same time period, the normal left hind paws showed no evidence of hyperalgesia. We therefore chose to expose rats to heat for 45 s in subsequently assessing the effects of agonists and antagonists.

In addition to the hyperalgesia induced by burn injury, PWLs under different stimulus intensities may not be equivalent. We found that a high stimulus intensity resulted in a lower difference between PWLs before and after burn injury than did a low stimulus intensity. We chose a low-intensity stimulus because we believed that the greater difference in latency could facilitate the assessment of hyperalgesia and the effects of analgesic drugs.

We showed that the intrathecally administered μ, δ, and α_2 adrenergic agonists, but not the κ agonist, attenuated the hyperalgesic state induced by mild burn injury. These effects were dose-dependent and, from the calculated

Table 1 ED10S and ED20S and their 95% CIs for dose–response curves of intrathecal agents

Agent	Paw	ED10S (95% CI) (μg)	ED20S (95% CI) (μg)
Morphine	Injured	0.31 (0.16–0.61)	17.5 (7.4–41.6)
	Normal	–	15.3 (6.0–39.2)
U50488H	Injured	>100	>100
	Normal	–	>100
DPDPE	Injured	27.59 (13.77–55.24)	2905 (3.7 to >10000)
	Normal	–	1805 (0.00 to >10000)
ST-91	Injured	1.60 (0.65–3.97)	179.3 (2.81 to >10000)
	Normal	–	94 (0.3 to >10000)

Data are presented as means ± SE. *Dash* indicates normal baseline

ED10S Effective dose required to raise the response latency to 10 s, *ED20S* effective dose required to raise the response latency to 20 s, *CI* confidence interval

Table 2 Effects of intrathecally administered opioid and α_2 antagonists without agonists on PWL of mild burn injury

Agent	*n*	PWL (s)		
		Normal	Injured	Difference
Control	6	13.1 ± 0.4	6.4 ± 0.3	−6.5 ± 0.4
Naloxone 30 μg	8	12.0 ± 0.4	7.1 ± 0.4	−5.0 ± 0.4
Naltrindole 30 μg	8	12.4 ± 0.4	7.5 ± 0.5	−4.9 ± 0.8
Yohimbine 30 μg	5	11.8 ± 0.4	6.2 ± 0.4	−5.6 ± 0.6

Data are presented as means ± SE

PWL Paw withdrawal latency

Table 3 Effects of intrathecally administered opioid and α_2 antagonists plus agonists on PWL of mild burn injury

Agent		*n*	PWL (s)		
Antagonist	Agonist		Normal	Injured	Difference
Control	Morphine 10 μg	6	19.1 ± 0.3	18.7 ± 0.5	−0.4 ± 0.5
	DPDPE 100 μg	6	16.6 ± 1.3	13.4 ± 1.9	−3.2 ± 1.6
	ST-91 10 μg	5	15.9 ± 0.8	13.9 ± 1.2	−2.0 ± 1.0
Naloxone 30 μg	Morphine 10 μg	4	11.4 ± 0.7*	7.5 ± 0.4*	−5.1 ± 0.6
	DPDPE 100 μg	4	12.5 ± 0.8*	7.4 ± 0.4*	−4.0 ± 0.6
Naltrindole 30 μg	Morphine 10 μg	4	18.5 ± 1.0	15.3 ± 1.3	−3.2 ± 0.6
	DPDPE 100 μg	4	11.2 ± 0.7*	8.5 ± 0.8*	−2.7 ± 1.4
Yohimbine 30 μg	ST-91 10 μg	5	11.6 ± 0.5*	6.7 ± 0.3*	−4.9 ± 0.5

Data are presented as means ± SE

PWL Paw withdrawal latency

* $P < 0.05$ compared with its respective control

ED10S and ED20S values, the order of drug potency in this hyperalgesia model was morphine > ST-91 > DPDPE > U50488H = 0. This result corresponds with previous reports for other nociceptive endpoints such as the tail-flick or phase1 formalin tests [5, 7, 21].

We found that the antagonist naloxone inhibited the effects of intrathecal morphine and DPDPE, and that naltrindole blocked the effects of DPDPE but not morphine. These results indicate that spinal μ and δ agonists can powerfully modulate the somatic responses to a noxious thermal stimulus via an opioid mechanism. Our findings are consistent with previous studies, which showed that the spinal action of μ and δ agonists produced powerful analgesia in rats [7]. Binding, electrophysiology, and transmitter release studies support the hypothesis that μ and δ opioids may act presynaptically to inhibit the release of peptide from at least the class of C fibers that release substance P and calcitonin-gene-related peptide (CGRP) [22].

In previous studies, intrathecally delivered δ agonists depressed the behavioral and electrophysiologic responses evoked by acute noxious stimuli such as the tail-flick and hot plate tests. On the other hand, intrathecal κ agonists suppressed the response to protracted pain which was typically induced by visceral chemical and inflammatory stimuli. The μ agonists produced powerful analgesia in conditions of both acute noxious stimuli and protracted pain [5, 7, 21, 23]. Opioid receptors have different distributions within the lumbar region of the spinal cord in rats. Both μ receptors and κ receptors have been localized to the superficial dorsal horn (laminae I–II), whereas δ receptors were found to be distributed throughout the entire dorsal horn (laminae I–VI) [24]. But the different distribution of receptors does not provide a sufficient explanation for the differential activity. Whether the relatively modest effect of the κ agonists reflects the lack of intrinsic activity in the spinal cord, or whether this effect represents a distinctive role for κ receptors in spinal nociceptive processing remains to be determined.

ST-91, a polar analog of clonidine, produces analgesia in normal rats and in rats after nerve injury, without significant hypotension, bradycardia, or sedation [6]. The favorable profile of ST-91 could reflect its range of distribution due to its hydrophilicity or due to receptor subtype selectivity [25]. Intrathecal administration of α_2 adrenoceptor agonists, such as dexmedetomidine, clonidine, and ST-91, has been found to produce antinociception in rats [6, 13, 26–29] by inhibiting synaptic transmission in the rat spinal cord dorsal horn [27, 30].

We also found that intrathecal ST-91 produced dose-dependent antinociceptive effects after mild burn injury and that these effects of ST-91 were reversed by yohimbine, which acts preferentially on α_2 adrenoceptors [31–33]. This finding indicates that, like opioid receptors, α_2 adrenoceptors powerfully modulate somatic responses to noxious thermal stimuli.

One advantage of the present mild burn injury model is that control animals are not required, because the non-injured left hind paw of each rat can serve as a control for the injured right hind paw. Measurements in the non-

injured hind paw were not affected by the mild burn injury in the contralateral hind paw or by the agonists and antagonists. The use of this model may reduce errors due to individual variability, as well as reducing the number of experimental rats, their costs, and the time required to perform experiments.

In summary, an adequate thermal hyperalgesia model was generated by placing a hot plate, at a temperature of 52.5 ± 1°C, in contact with the hind paw of a rat for 45 s and by checking PWL using a 5.0-*A* heat stimulus. In this experimental model, μ, δ, and α_2 receptor agonists, but not a κ receptor agonist, produced a dose-dependent antinociceptive effect at the level of the spinal cord. Further research, using drugs associated with pain transmission, is needed to clarify the mechanism of hyperalgesia induced by mild burns.

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