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Clonidine, but Not Morphine, Delays the Development of Thermal Hyperesthesia Induced by Sciatic Nerve Constriction Injury in the Rat

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Background: **It has been shown that the spinal facilitation induced by the injury discharge evoked by a nerve constriction injury is crucial in the development of thermal hyperesthesia. Both opioids and α_2 agonists have been reported to prevent the development of spinal facilitation evoked by the small afferent input to the spinal cord. Moreover, it has been reported that the thermal hyperesthesia induced by a nerve constriction injury is sympathetically maintained and that spinally administered α_2 agonists can modulate the sympathetic outflow from the spinal cord. The current study investigated the effect of spinally administered morphine and clonidine, an α_2 agonist, on the development of thermal hyperesthesia induced by nerve constriction injury in the rat.**

Methods: **A model of thermal hyperesthesia induced by a constriction injury created by making four loose ligatures around the rat sciatic nerve was used to examine the development of thermal hyperesthesia. Morphine, clonidine, and idazoxan were administered intrathecally or intraperitoneally 20 min before (pretreatment study) or 20 min after (posttreatment study) the nerve injury.**

Results: **Pretreatment, but not posttreatment, with intrathecal clonidine significantly delayed the development of thermal hyperesthesia in a dose-dependent manner, and this delay in onset produced by clonidine was 3 days after the nerve injury. This effect of clonidine's was completely antagonized by the coadministration of idazoxan with clonidine. Intrathecal morphine had no effect on the development of thermal hyperesthesia in this study.**

Conclusion: **Spinal α_2 receptors, but not opioid receptors, may play an important role in the development of thermal hyperesthesia induced by a nerve constriction injury. This suggested that the activation of spinal α_2 receptor may reduce the sympathetic outflow and this reduction of sympathetic outflow may be the key mechanism that delays the development of thermal hyperesthesia. (Key words: Analgesics, opioid: morphine. Pain: neuropathic. Sympathetic nervous system, α_2-adrenergic agonists: clonidine.)**

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SENSORY nerve damage may induce neuropathic pain that is constant, intermittent, or paroxysmal with a burning, sharp, or aching sensation. Damage to the sensory nerve induces a barrage of impulses (injury discharge).[1] It has been suggested that this injury discharge may induce the N-methyl-D-aspartate (NMDA) receptor-dependent facilitation of spinal dorsal horn neurons, and this spinal facilitation may play an important role in the development of neuropathic pain.[2-4] For example, blocking injury discharge by topical application of local anesthetics or blocking spinal facilitation by intrathecal administration of NMDA receptor antagonists delays the development of neuropathic pain.[2]

It has been reported that both opioids and α_2 agonists modulate the input to the spinal cord evoked by small primary afferents in a similar manner. Both opioid and α-adrenergic receptors are situated both presynaptic and postsynaptic to the small primary afferents.[5,6] Both opioids and α_2 agonists diminish the release of peptides believed to originate from small primary afferents,[7,8] and single-unit recording of spinal dorsal horn neurons revealed that spinal μ and α_2 agonists reduce the discharges evoked by acute activation of small, but not large, primary afferents.[9,10] Both opioids and α_2 agonists have been shown to block the development of spinal facilitation evoked by the nociceptive input to the spinal cord when the drugs were preemptively administered.[11-13] These data suggest that both opioids and α_2 agonists, administered intrathecally just before the nerve injury, may modify the hyperexcitability level of the spinal dorsal horn neurons that was induced by injury discharge, by the modulative effect of opioids and α_2 agonist on the nociceptive input to the spinal cord. Conversely, it has been reported that sciatic nerve section caused a significant ipsilateral increase in c-Fos-like immunoreactivity in the dorsal horn in the rat and that intrathecal morphine, but not clonidine, prevented the expression of c-Fos-like immunoreactiv-

ity after axotomy in the dorsal horn when morphine or clonidine was administered before the sciatic nerve section.[14] These data suggested that the effect of morphine on the spinal dorsal horn neuron after the nerve injury was different from that of clonidine.

Recently, it has been shown that ipsilateral thermal and mechanical hyperesthesia occur 5–7 days after the constriction injury created by making four loose ligatures around the sciatic nerve in the rat.[15,16] This thermal hyperesthesia induced by the constriction nerve injury is reported to be sympathetically maintained.[17] If both opioids and α_2 agonists, administered intrathecally just before the nerve constriction injury, prevent the development of spinal facilitation evoked by injury discharge, then both opioids and α_2 agonists may prevent the development of thermal hyperesthesia induced by the nerve constriction injury.

In the current study, therefore, we sought to define the effect of intrathecally or intraperitoneally administered morphine and clonidine on the development of thermal hyperesthesia induced by sciatic nerve constriction injury in the rat.

Methods

The following investigations were carried out under the protocol approved by the Institutional Animal Care Committee at Chiba University. Male Sprague-Dawley rats (weighing 250–300 g) were prepared with intrathecal catheters and examined for the effects of agents on the development of thermal hyperesthesia induced by the nerve constriction injury.

Intrathecal Catheters

Chronic intrathecal catheters were inserted, during isoflurane anesthesia, by passing a PE-10 catheter through an incision in the atlantooccipital membrane to a position 8 cm caudal to the cisterna at the level of the lumbar enlargement.[18] 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. Rats showing postoperative neurologic deficits were discarded.

Nerve Constriction Injury

The hyperesthetic state was induced by chronic constriction of the sciatic nerve with four loose ligatures.[15] Anesthesia was induced by inhalation of 5% isoflurane, maintained at a concentration of 2% or 3% as needed. After the local incision, the biceps femoralis of each leg was bluntly dissected at mid-thigh to expose the sciatic nerve. Each nerve was then carefully mobilized, with care taken to avoid undue stretching. Each of four 4-0 chromic gut sutures was then tied loosely with a square knot around the right sciatic nerve. The left sciatic nerve was only mobilized. Both incisions were closed, layer to layer, with 3-0 silk sutures, and the rats were allowed to recover from anesthesia. After receiving the sciatic nerve constriction injury, the animals were maintained individually in clear plastic cages with solid floors covered with 3–6 cm sawdust. Animals appropriately prepared showed a mild to moderate degree of foot drop (*i.e.*, weakness of the hind paw's dorsiflexors). All animals displayed normal feeding and drinking postoperatively.

Thermal Nociceptive Test

Paw withdrawal latency (PWL) against thermal stimulation was measured with a device similar to that previously reported.[19] The rats were placed beneath a clear plastic cage (10 × 20 × 24 cm) on an elevated floor of clear glass (2-mm thick). A radiant heat source (eye projector halogen lamp JRC-12V-100W, Iwasaki Electric, Tokyo, Japan) with an aperture diameter of 5 mm was contained in a movable holder placed beneath the glass floor. The voltage to the thermal source was controlled by a constant voltage supply. To reduce the variability in plate surface temperature resulting from minor changes in room temperature, the interior of the box under the animal was prepared with a heat source such that glass temperature was regulated at 30°C. The calibration of the thermal test system is such that the average response latency in ten normal untreated rats was maintained at 10 s before the initiation of an experiment series.

To initiate a test, a rat was placed in the box and allowed 5–10 min to habituate. The halogen lamp beneath the floor was then positioned so that it focused on the plantar surface of one hind paw that was in contact with the glass plate. The light was then activated, initiating a timing circuit. The interval between the application of the light beam and the brisk hind paw withdrawal response was measured to the nearest 0.1 s. The trial was terminated and the lamp removed in the absence of a response within 20 s. The interval was then assigned as the response latency.

Behavioral Analysis

The general behavior of each rat was carefully observed and tested. Motor function was evaluated by

the performance of two specific behavioral tasks.[20] (1) Placing-stepping reflex: this response was evoked by drawing the dorsum of either hind paw over the edge of a table top. In normal animals this stimulus elicits an upward lifting of the paw onto the surface of the table (stepping). (2) Righting reflex: an animal placed horizontally with its back on the table normally shows an immediate coordinated twisting of the body around its longitudinal axis to regain its normal position on its feet. Animals displaying ataxic behavior show a decreased ability to right themselves. To quantitate the extent of motor function, both tasks were scored on a scale of 0–2, where 0 = absence of function and 2 = normal motor function. Animals that were able to perform the motor tasks but more slowly than normal animals were assigned a score of 1.

Experimental Protocol

Effects of Drugs on the Thermal Nociceptive Test in Normal Rats. Before the intrathecal drug injection, both right and left hind PWL against thermal stimuli was measured alternately three times, with 5-min intervals between each testing of one paw, to generate the baseline data. Then, morphine or clonidine was administered intrathecally, and the left and right hind paws were tested at 5, 15, 30, 60, and 90 min after the intrathecal drug injection. The average of the right and left hind PWLs was defined as the PWL.

Effects of Drugs on the Development of Thermal Hyperesthesia. Before induction of the sciatic nerve injury, the right and left hind paws were tested three times alternately, with 5-min intervals allowed between consecutive tests, to obtain baseline data. The average of three measurements was defined as the PWL. The investigator who measured PWLs was blinded throughout the study to the treatment that the animal had received. Morphine or clonidine was administered intrathecally 20 min before (pretreatment study) or 20 min after (posttreatment study) the loose ligatures were made. The postsurgery PWLs of the right and left hind paws were measured 3, 7, and 14 days after the nerve lesion.

An additional group of rats was used to verify that the effect of clonidine on the development of thermal hyperesthesia induced by the nerve constriction injury was produced by an interaction between clonidine and α_2 receptors. In this group of rats, 300 μg idazoxan, an α_2 receptor antagonist, was coadministered with 100 μg clonidine 20 min before the nerve constriction injury. To prove whether 300 μg idazoxan itself has some effect on the development of thermal hyperesthesia, 300 μg idazoxan was administered intrathecally 20 min before the nerve constriction injury. The postsurgery PWLs of right and left hind paws of the additional groups were measured 3, 7, and 14 days after the nerve lesion.

To verify that the effect of intrathecally administered clonidine on the development of thermal hyperesthesia evoked by the nerve constriction injury was produced by an interaction between clonidine and spinal α_2 receptor, 100 μg clonidine or saline was administered intraperitoneally 20 min before the nerve injury. The postsurgery PWLs of right and left hind paws were measured 3, 7, and 14 days after the nerve injury.

It has been reported that intrathecally administered clonidine, but not morphine, produced hypotension.[21–23] It is possible that the effect of intrathecally administered clonidine on the development of thermal hyperesthesia evoked by the nerve injury is due to hypotension. Intrathecally administered clonidine (1–30 μg) has been reported to result in a dose-dependent reduction in arterial blood pressure and 30 μg clonidine decreased the mean arterial blood pressure to about 50% of the predrug value in the 0.9% halothane-anesthetized rat.[22] When 60 μg clonidine was administered intrathecally, the magnitude of the hypotension was diminished.[22] Isoflurane (2.0 minimum alveolar concentration) was reported to decrease the mean arterial blood pressure to about 50% of the awake value in humans.[24] The minimum alveolar concentration value for isoflurane in rats was 1.46%.[25] Thus, we kept the animals anesthetized with 3% isoflurane anesthesia for 30 min after the nerve lesion, and the postsurgery PWLs of right and left hind paws were measured 3, 7, and 14 days after the nerve lesion. To obtain control data, we tested an additional group. In this group of rats, isoflurane (3%) anesthesia was discontinued at the end of the surgery for the nerve constriction injury.

Drugs

The agents used in this study were morphine hydrochloride (molecular weight = 322; Takeda, Osaka, Japan), clonidine hydrochloride (molecular weight = 267; Research Biochemicals, Natick, MA), and idazoxan hydrochloride (molecular weight = 241; Sigma, St. Louis, MO). All agents were dissolved in normal saline and administered intrathecally in 10 μl vehicle or intraperitoneally in 0.5 ml vehicle.

Data Analysis

Effects of Drugs on the Thermal Nociceptive Test in Normal Rats. To analyze the effect of intrathecally administered drugs on the thermal nociceptive test, the maximum PWL (MAX PWL) was calculated. MAX PWL was defined as the single longest PWL value during the first 30 min after the intrathecal drug injection. To obtain a dose-response curve, the dose was plotted against the MAX PWL. Dose-response curves were established with a least squares linear regression analysis. To evaluate the dose-dependence, the correlation coefficient was calculated.

Effects of Drugs on the Development of Thermal Hyperesthesia. To analyze the magnitude of the thermal hyperesthesia, the difference score (DS) was calculated by subtracting the PWL of the control side (left side) from the PWL of the injured side (right side). A negative score thus indicates a faster withdrawal latency to thermal nociceptive stimuli on the injured side, *i.e.*, hyperesthesia. The level of thermal hyperesthesia and the PWLs of the rats in each group before surgery (day 0) and on days 3, 7, and 14 were compared by one way analysis of variance (ANOVA). For multiple comparisons, we used Dunnett's test. To compare the PWLs and DSs before surgery (day 0) and on day 3, 7, and 14 between groups, ANOVA or Student's *t* test was used. To evaluate the dose-dependence, we calculated the correlation coefficient.

Wherever appropriate, results are expressed as means ± SEM. Critical values that reached a $P < 0.05$ level of significance were considered statistically significant.

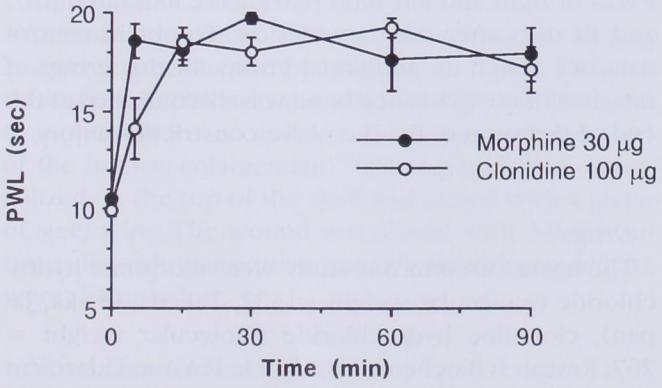

Fig. 1. Effects of intrathecal morphine (30 μg) and clonidine (100 μg) on the thermal nociceptive test in the normal rats. Ordinate: paw withdrawal latency. Abscissa: time (min) after drug injection. Each line represents the mean ± SEM determination made in four or five rats.

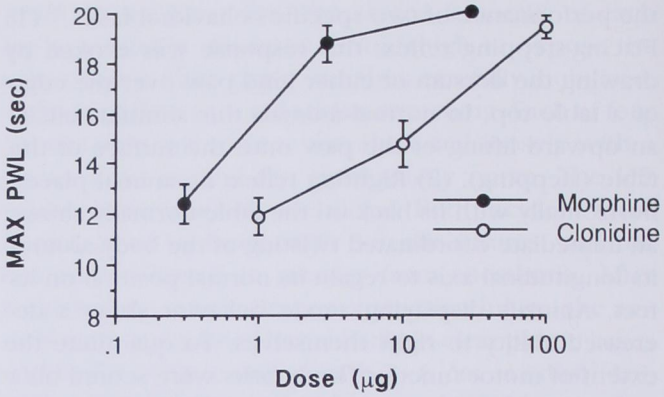

Fig. 2. Log dose-response curve for the effect of morphine and clonidine on maximum paw withdrawal latency (MAX PWL) in normal rats, where MAX PWL = the single longest paw withdrawal latency value during the first 30 min after intrathecal administration of the drug. Ordinate: MAX PWL; abscissa: log dose (μg). Each point represents the mean ± SEM of four or five rats.

Results

The animals that received 100 μg intrathecal morphine showed touch-evoked agitation behavior. Intrathecal injection of 30 μg morphine had no effect on the behavioral test. Thus, 30 μg morphine was the largest dose employed in the current study. Intrathecally administered clonidine and idazoxan had no effect on the placing, stepping, or righting reflexes throughout this experiment.

Effects of Drugs on the Thermal Nociceptive Test in Normal Rats

The mean baseline PWL in the thermal nociceptive test was 10.2 ± 0.1 s (n = 26). Either 30 μg morphine or 100 μg clonidine resulted in a significant increase in the PWL (fig. 1). The maximum effect of 30 μg morphine was obtained 5 min after the drug injection (MAX PWL = 20.0 ± 0 s, n = 5) and lasted at least until 90 min after the drug injection. The maximum effect of 100 μg clonidine was obtained 15 min after the drug injection (MAX PWL = 19.4 ± 0.5 s, n = 4) and lasted at least until 90 min after the drug injection.

Intrathecally administered morphine increased PWLs in a dose-dependent manner at a dose between 0.3 and 30 μg ($r = 0.89$, $P < 0.0001$; fig. 2), and intrathecally administered clonidine increased PWLs in a dose-dependent manner at a dose between 1 and 100 μg ($r = 0.91$, $P < 0.0001$; fig. 2).

Table 1. Presurgery Right and Left Paw Withdrawal Latency and Difference Scores in the Pretreatment Study

	Right PWL (s)	Left PWL (s)	DS (s)
Morphine 30 μg IT group (n = 11)	10.0 ± 0.4	10.3 ± 0.3	−0.3 ± 0.3
Clonidine 100 μg IT group (n = 11)	10.5 ± 0.3	10.2 ± 0.3	0.3 ± 0.1
Clonidine 30 μg IT group (n = 8)	10.0 ± 0.2	10.2 ± 0.3	−0.1 ± 0.1
Clonidine 10 μg IT group (n = 8)	10.4 ± 0.2	10.3 ± 0.3	0.0 ± 0.5
Clonidine + idazoxan IT group (n = 8)	10.3 ± 0.3	10.3 ± 0.3	0.0 ± 0.2
Idazoxan IT group (n = 8)	10.2 ± 0.4	10.3 ± 0.4	−0.1 ± 0.1
Saline IT group (n = 12)	10.1 ± 0.3	10.3 ± 0.3	−0.2 ± 0.1
Clonidine 100 μg IP group (n = 9)	10.2 ± 0.4	10.2 ± 0.2	0.0 ± 0.2
Saline IP group (n = 9)	9.9 ± 0.2	9.8 ± 0.2	0.1 ± 0.1
3% isoflurane 30 min inhalation group (n = 8)	10.0 ± 0.2	10.2 ± 0.3	−0.2 ± 0.2
3% isoflurane inhalation during surgery (n = 8)	10.0 ± 0.2	10.2 ± 0.2	−0.1 ± 0.1

Values are mean ± SEM.

PWL = paw withdrawal latency; DS = difference score; IT = intrathecal; IP = intraperitoneal.

Effects of Drugs on the Development of Thermal Hyperesthesia

Pretreatment Study. Table 1 shows the presurgery right and left PWLs and DSs in each group. Analysis of variance showed that there were no difference between the presurgery right and left PWLs and DSs of each group (right PWL $P > 0.9$; left PWL $P > 0.9$; DS $P > 0.2$).

In each group, there were no differences between the left PWLs on day 0, 3, 7, and 14 after the nerve constriction injury ($P > 0.05$, ANOVA, tables 1 and 2). In the saline-treated rats, the DSs on days 3, 7, and 14 after the nerve constriction injury were significantly more negative than the presurgical values ($P < 0.05$, ANOVA; fig. 3). In the rats treated with 30 μg morphine, the DSs on days 3, 7, and 14 were more negative than the presurgical values ($P < 0.05$, ANOVA). The DSs on days 3 and 14 in the rats treated with 30 μg morphine were not different from those in the rats treated with saline ($P > 0.5$, t test), but the DS on day 7 in the rats treated with 30 μg morphine was more negative than that in the rats treated with saline ($P < 0.05$, t test; fig. 3).

When clonidine was administered intrathecally 20 min before the nerve constriction injury, clonidine attenuated the level of thermal hyperesthesia induced by the nerve constriction injury in a dose-dependent manner on days 3 ($r = 0.73$, $P < 0.0001$) and 7 ($r = 0.40$, $P < 0.05$) at a dose between 10 and 100 μg (fig. 4). On day 14 after the nerve constriction injury, pretreatment of intrathecal clonidine had no effect on the level of thermal hyperesthesia at a dose between

Table 2. Postsurgery Left Paw Withdrawal Latency in the Pretreatment Study

	Day 3 (s)	Day 7 (s)	Day 14 (s)
Morphine 30 μg IT group (n = 11)	10.9 ± 0.3	10.8 ± 0.2	10.6 ± 0.3
Clonidine 100 μg IT group (n = 11)	10.1 ± 0.2	10.9 ± 0.3	10.7 ± 0.2
Clonidine 30 μg IT group (n = 8)	11.0 ± 0.4	10.7 ± 0.2	10.6 ± 0.4
Clonidine 10 μg IT group (n = 8)	10.8 ± 0.3	10.3 ± 0.4	10.2 ± 0.5
Clonidine + idazoxan IT group (n = 8)	11.2 ± 0.4	10.6 ± 0.3	10.1 ± 0.2
Idazoxan IT group (n = 8)	10.4 ± 0.3	10.4 ± 0.3	10.4 ± 0.1
Saline IT group (n = 12)	11.3 ± 0.3	10.3 ± 0.3	10.5 ± 0.4
Clonidine 100 μg IP group (n = 9)	10.5 ± 0.2	10.3 ± 0.2	10.3 ± 0.2
Saline IP group (n = 9)	10.5 ± 0.4	10.7 ± 0.3	10.5 ± 0.3
3% isoflurane 30 min inhalation group (n = 8)	10.7 ± 0.3	10.4 ± 0.2	10.2 ± 0.2
3% isoflurane inhalation during surgery (n = 8)	10.0 ± 0.2	9.9 ± 0.3	10.3 ± 0.2

Values are mean ± SEM.

IT = intrathecal; IP = intraperitoneal.

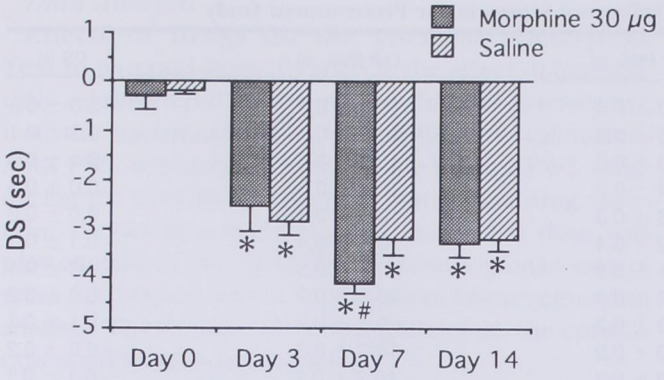

Fig. 3. Effect of 30 μg intrathecal morphine (n = 11) and saline (n = 12) on the development of thermal hyperesthesia induced by a nerve constriction injury in the pretreatment study. Each bar represents the mean ± SEM. DS = difference score. *$P < 0.05$ compared with presurgery (day 0) value in the same group. #$P < 0.05$ compared with the saline group 7 days after the nerve injury.

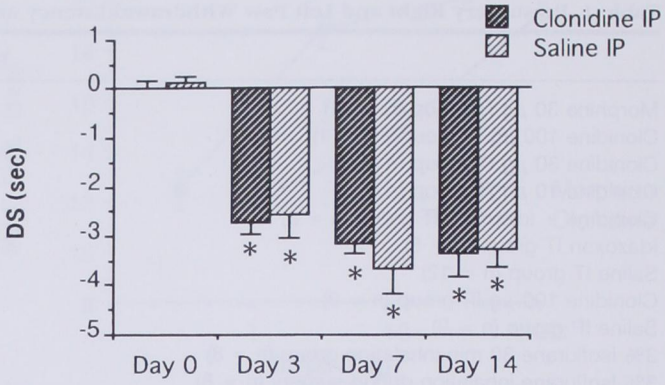

Fig. 5. Effect of 100 μg intraperitoneal clonidine (n = 9) and saline (n = 8) on the development of thermal hyperesthesia induced by a nerve constriction injury in the pretreatment study. Each bar represents the mean ± SEM. DS = difference score. *$P < 0.05$ compared with presurgery (day 0) value in the same group.

10 and 100 μg (r = 0.06, $P > 0.7$; fig. 4). When 100 μg clonidine was administered intraperitoneally, the DSs on days 3, 7, and 14 were not significantly different from those in the rats injected intraperitoneally with saline ($P > 0.3$, *t* test; fig. 5).

When 300 μg idazoxan was coadministered with 100 μg clonidine 20 min before the nerve constriction injury, the DSs on days 3 and 7 were more negative than those in the 100 μg clonidine-treated animals (day 3: $P < 0.001$, day 7: $P < 0.05$, *t* test) and the DS on day 14 was not significantly different from that of 100 μg clonidine-treated rats ($P > 0.7$, *t* test; fig. 6). In the 300 μg idazoxan-pretreated rats, the DSs on days 3, 7, and 14 after the nerve injury were not significantly different from those in the saline-treated rats ($P > 0.1$, *t* test; fig. 6).

In both rats anesthetized with 3% isoflurane anesthesia for 30 min after the nerve injury and in rats anesthetized with 3% isoflurane anesthesia only during the surgery, the DSs on days 3, 7, and 14 were more negative than the presurgery values ($P < 0.05$, ANOVA; fig. 7). The DSs on days 3, 7, and 14 of the rats that were

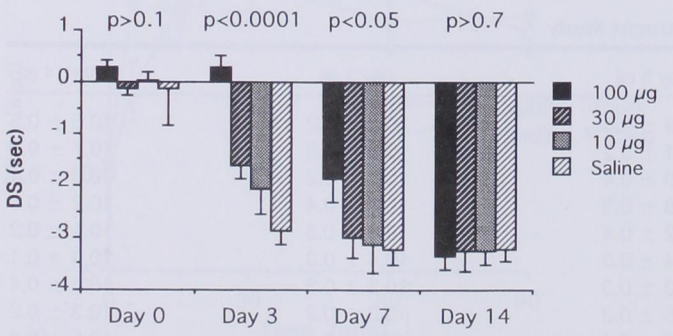

Fig. 4. Dose-response analysis of the effect of clonidine on the difference score (DS) at 3, 7, and 14 days after the nerve injury. Each bar shows the group mean ± SEM of 8–11 rats. The saline-administered group (n = 12) is presented for comparison. *P* value was at the significant level when analyzed by the correlation coefficient.

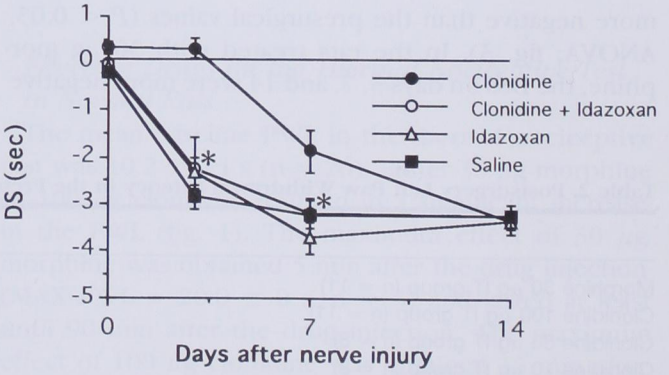

Fig. 6. Effect of intrathecal coadministration of 300 μg idazoxan with 100 μg clonidine and intrathecal administration of 300 μg idazoxan on the development of thermal hyperesthesia induced by a nerve constriction injury in the pretreatment study. Clonidine (100 μg)-administered group (n = 11) and saline-administered group (n = 12) are presented for comparison. Each point represents the mean ± SEM of eight rats. Ordinate = difference score (DS); abscissa = days after a nerve constriction injury. *$P < 0.05$ compared DS value in 100-μg clonidine and 300-μg idazoxan coadministered group with that in the 100-μg clonidine administered group.

kept under 3% isoflurane anesthesia for 30 min after the surgery of nerve constriction injury were not significantly different from those in the rats anesthetized with 3% isoflurane only during surgery ($P > 0.4$, t test; fig. 7).

Posttreatment Study. In both the saline-treated (n = 9) and 100 μg-treated rats (n = 9), the DSs on days 3, 7, and 14 after the nerve injury were significantly more negative than the presurgical value ($P > 0.5$, ANOVA; fig. 8). On days 3, 7, and 14, the DSs of the 100 μg clonidine-treated rats were not significantly different from those in the saline-treated rats ($P > 0.1$, t test; fig. 8).

Discussion

The current study clearly demonstrated that intrathecally administered clonidine, but not morphine, delayed the development of thermal hyperesthesia induced by the nerve constriction injury, when clonidine was administered 20 min before, but not after, the nerve injury. The effect of clonidine was dose-dependent and was completely antagonized with idazoxan when idazoxan was coadministered with clonidine. This delay in onset produced by pretreatment of clonidine was 3 days after the nerve injury and, 14 days after the nerve injury, the level of thermal hyperesthesia in

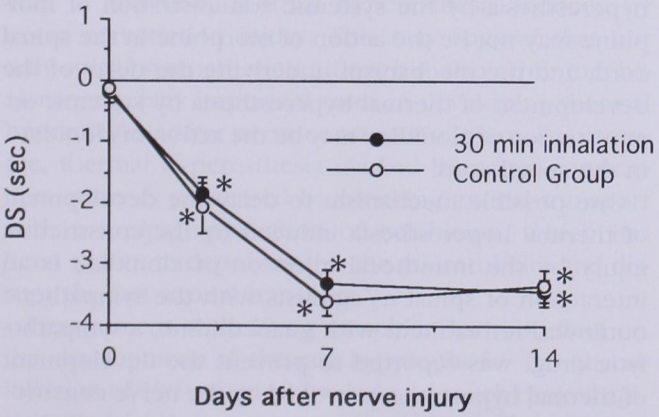

Fig. 7. Effect of 3% isoflurane inhalation on the development of thermal hyperesthesia induced by a nerve constriction injury. Each point represents the mean ± SEM. Thirty-min inhalation group (n = 8): anesthetized with 3% isoflurane for 30 min after the nerve injury; control group (n = 8): isoflurane (3%) anesthesia was discontinued at the completion of the surgery for the nerve constriction injury. Ordinate = difference score (DS); abscissa = days after a nerve constriction injury. *$P < 0.05$ compared with presurgery (day 0) value in the same group.

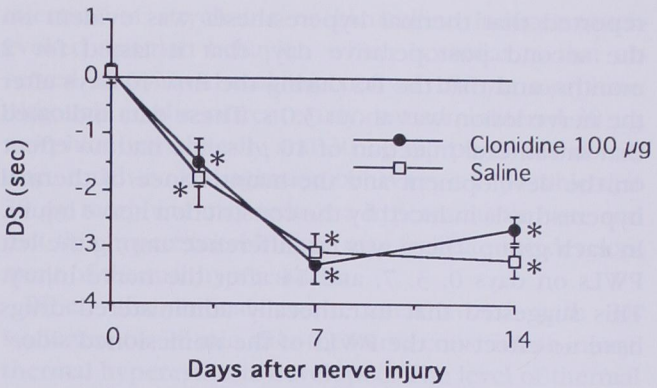

Fig. 8. Effect of 100 μg intrathecal clonidine and saline on the development of thermal hyperesthesia induced by a nerve constriction injury in the posttreatment study. Each point represents the mean ± SEM of nine rats. Ordinate = difference score (DS); abscissa = days after a nerve constriction injury. *$P < 0.05$ compared with presurgery (day 0) value in the same group.

the clonidine-pretreated animals was the same as that in the saline-pretreated rats. Moreover, intraperitoneal injection of clonidine had no effect on the development of thermal hyperesthesia at the dose that delayed the development of thermal hyperesthesia when administered intrathecally. These data suggest that the activation of spinal α_2 receptors delayed the development of thermal hyperesthesia after the nerve constriction injury. Idazoxan itself had no effect on the development of thermal hyperesthesia at the dose that antagonized the effect of intrathecally administered 100 μg clonidine when coadministered with 100 μg clonidine 20 min before the nerve constriction injury. This suggested that spinal α_2 receptors were not tonically activated.

To evaluate the behavioral function, we used the placing-stepping reflex and the righting reflex. In these reflexes, tactile/proprioceptive input is involved. It has been reported that both intrathecally administered opioids and α_2 agonists inhibited the spinal nociceptive input, but not tactile/proprioceptive input.[5,6] Thus, we think that these two reflexes are appropriate to analyze the behavioral function in the current study.

Time Course of the Development of Thermal Hyperesthesia

In the saline-treated animals, thermal hyperesthesia developed 3 days after the nerve injury. Maximum thermal hyperesthesia (DS approximately 3.0 s) occurred 7–14 days after the nerve injury. Bennett and Xie[15]

reported that thermal hyperesthesia was evident on the second postoperative day, that it lasted for 2 months, and that the DS during the first 40 days after the nerve lesion was about 3.0 s. These data indicated that intrathecal injection of 10 μl saline had no effect on the development and the maintenance of thermal hyperesthesia induced by the constriction nerve injury. In each group, there was no difference among the left PWLs on days 0, 3, 7, and 14 after the nerve injury. This suggested that intrathecally administered drugs have no effect on the PWLs of the nonlesioned side.

Role of Opioid and α_2 Receptors on the Development of Thermal Hyperesthesia

Both morphine and clonidine were administered intrathecally 20 min before the nerve constriction injury in the pretreatment study. Time course of the thermal nociceptive test revealed that the maximum effects were obtained within the first 15 min after the intrathecal administration of morphine or clonidine and the maximum effects lasted, at least, until 90 min after the drug injection. These data indicated that, in the pretreatment study, nerve constriction injury was performed at a time such that maximum antinociceptive effects were obtained. Thus, we think that 20 min before the nerve constriction injury was the appropriate time for the drug injection to define the effect of preemptively administered morphine and clonidine on the development of thermal hyperesthesia induced by the nerve constriction injury.

Both intrathecally administered morphine and clonidine increased PWLs in a dose-dependent manner in the thermal nociceptive test. MAX PWL in the 30 μg morphine-treated rats is 20 s and this value is almost the same as that in the 100 μg clonidine-treated rats (19.4 s). As mentioned earlier, both opioids and α_2 agonists block the neuronal transmission to the spinal dorsal horn neuron from small primary afferents fibers in a similar manner and produce antinociceptive effect in the thermal nociceptive test. Conversely, pretreatment with 100 μg clonidine delayed the development of thermal hyperesthesia induced by the constriction nerve injury and pretreatment with 30 μg morphine had no effect on the development of thermal hyperesthesia. Thus, the mechanisms underlying the delayed effect of 100 μg clonidine on the development of thermal hyperesthesia are different from the ability of clonidine to modulate the input to the spinal dorsal horn neurons evoked by small primary afferents.

It has been reported that pretreatment with an NMDA receptor antagonist blocked the development of spinal facilitation evoked by the constriction nerve injury and delayed the development of thermal hyperesthesia.[2] Both opioids and α_2 agonists inhibit the capsaicin-evoked release of glutamate from rat spinal dorsal horn slices.[26,27] Glutamate is present in the terminals of small-diameter primary afferent fibers, as well as in dorsal horn interneurons,[28,29] and capsaicin-evoked release of glutamate is thought to originate mainly from primary afferents.[30] It has been reported that the NMDA receptor is not located postsynaptic to primary afferent input; rather it mediates excitation evoked by glutamate-releasing interneurons.[31] Thus, we think that both opioids and α_2 agonists are not able to block the activation of NMDA receptor in the spinal cord. This is consistent with the report that spinally mediated facilitation of the dorsal horn neurons evoked by repetitive input from C-fibers (wind-up phenomena[32]) is blocked by NMDA receptor antagonist, and that opioids appear to poorly modulate this central facilitation.[33,34]

It has been reported that pretreatment with systemic morphine or clonidine prevents the development of mechanical hyperesthesia in the nerve constriction injury model.[35] In the current study, intrathecal clonidine, but not intrathecal morphine, delayed the development of thermal hyperesthesia induced by the nerve constriction injury. This suggests that the mechanism underlying the delay of the development of mechanical hyperesthesia by the systemic administration of morphine may not be the action of morphine in the spinal cord, and the mechanism underlying the delay of the development of thermal hyperesthesia by systemic administration of clonidine may be the action of clonidine in the spinal cord.

One possible mechanism to delay the development of thermal hyperesthesia induced by the constriction injury by the intrathecal injection of clonidine is an interaction of spinal α_2 agonists with the sympathetic outflow. Pretreatment with guanethidine, a sympatholytic drug, was reported to prevent the development of thermal hyperesthesia evoked by the nerve constriction injury,[17] and these data suggested that a sufficient level of sympathetic outflow is required for the development of thermal hyperesthesia induced by the nerve constriction injury. Intrathecal injection of clonidine produced decreases in the neural activity recorded from the lumbar sympathetic chain and caused changes in rat thermoregulation consistent with a reduction in efferent sympathetic activity.[36] Intrathecal clonidine

administration reduces adrenal medullary secretion.[37] Intrathecally administered α_2 agonists produce a dose-dependent decrease in blood pressure[21,22] and this effect is believed to be mediated by a direct effect on thoracolumbar neurons.[38] These data suggest that intrathecally administered α_2 agonists can reduce sympathetic outflow by direct action on the sympathetic preganglionic neurons. Thus, we think that intrathecal clonidine decreases the sympathetic outflow from the spinal cord and this reduction of sympathetic outflow delays the development of thermal hyperesthesia induced by the constriction nerve injury in the current study. We have previously reported that, when opioids or α_2 agonists were administered intrathecally 1 or 2 weeks after a nerve constriction injury, both opioids and α_2 agonists had no specific effect on the level of thermal hyperesthesia.[39] This may suggest that the mechanism underlying the development of thermal hyperesthesia after the constriction injury is different from that underlying the maintenance of thermal hyperesthesia. Wakisaka *et al.*[40] reported that there was a gradual loss of norepinephrine-containing sympathetic efferents in the constriction nerve injury model and that the decrease was first noted 5 days after the nerve injury and was very marked at 10–14 days after the nerve injury. This indicated that the role of sympathetic nerves in the maintenance of thermal hyperesthesia may change in the time-dependent manner.

Pretreatment of clonidine delays, but does not prevent or diminish, thermal hyperesthesia induced by nerve constriction injury. This may suggest that not only spinal sympathetic outflow but also other mechanisms may play important roles in developing thermal hyperesthesia after nerve constriction injury. For example, thermal hyperesthesia evoked by nerve constriction injury is prevented by blocking axonal transport with a topical application of colchicine, which has been shown to depolymerize the microtubules and disrupt the fast axonal transport.[41]

Pretreatment with 30 μg morphine had no effect on the development of thermal hyperesthesia. Intrathecal injection of 100 μg morphine induced touch evoked agitation behavior, and this suggested that a high dose of morphine, but not clonidine, may induce a hyperexcitable state in the spinal cord. We previously reported that an intrathecal injection of either strychnine or bicuculline enhanced the magnitude of the thermal hyperesthesia induced by the nerve constriction injury when strychnine or bicuculline was administered just after the nerve lesion and on day 1 and day 2 after the nerve lesion.[42] Intrathecal injection of strychnine or bicuculline induces touch-evoked agitation behavior, which is similar to that observed after intrathecal injection of 100 μg morphine. Although, in the current study, we administered 30 μg morphine intrathecally and 30 μg morphine did not induce touch-evoked agitation behavior, it is possible that 30 μg morphine may induce a hyperexcitable state and that this hyperexcitable state may facilitate the development of thermal hyperesthesia.

When the animals were anesthetized with 3% isoflurane for 30 min after the nerve constriction injury, thermal hyperesthesia developed. The level of thermal hyperesthesia in the rats that were anesthetized with 3% isoflurane for 30 min after the nerve injury is the same as that in the rats that were kept under 3% isoflurane anesthesia only during the surgery for the nerve constriction injury. In the posttreatment study, clonidine was administered 20 min after the nerve injury, and clonidine had no effect on the development of thermal hyperesthesia. This indicated that the 20 min posttreatment with clonidine is outside the effective window. Thus, we kept animals under isoflurane anesthesia 30 min after the nerve injury and kept animals in hypotension for 30 min after the nerve injury. These data suggested that hypotension itself had no effect on the development of thermal hyperesthesia induced by nerve constriction injury. It has been reported that 1.5 minimum alveolar concentration isoflurane does not alter the sympathetic nerve activity.[43] The processes leading to spinal facilitation, such as wind-up phenomena, are not substantially obtunded by volatile anesthetics.[34,44] It is reasonable that isoflurane does not decrease the sympathetic outflow and does not suppress the process leading to spinal facilitation evoked by the nerve constriction injury and that isoflurane does not delay the development of thermal hyperesthesia induced by the nerve constriction injury. It has been reported that volatile anesthetics enhance the suppressive effect of opioid analgesics on the spinal facilitation process evoked by nociceptive simulation.[45] Thus, it is possible the interaction of clonidine and isoflurane may contribute to the delayed effect of clonidine on the development of thermal hyperesthesia in the pretreatment study.

Our results suggest that preemptively administered clonidine may prevent the development of neuropathic pain. Recently it has been reported that epidural clonidine can modulate neuropathic pain or sympathetically maintained pain in humans.[46,47] Additional systematic clinical studies are required.

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