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Activation of spinal orexin-1 receptor produces anti-allodynic effect in the rat carrageenan test

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Abstract

Orexin-A and orexin-1 receptors are found in the dorsal root ganglion cells and the spinal dorsal horn and this suggests that orexin-A is involved in the spinal nociceptive transmission. The authors examined the effect of intrathecally administered orexin-A on the level of mechanical allodynia and thermal hyperalgesia induced by paw carrageenan injection in the rat. Intrathecal injection of 0.3 and 3 nmol of orexin-A suppressed the level of mechanical allodynia, but not that of thermal hyperalgesia, and the effect of orexin-A on mechanical allodynia was antagonized by the pretreatment of 1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride, SB-334867, a selective orexin-1 receptor antagonist. These data suggest that the activation of spinal orexin-1 receptor modulates the mechanical information transmission, but not thermal information transmission, in the spinal cord during carrageenan test.
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Keywords: Orexin-A; Mechanical allodynia; Thermal hyperalgesia; SB-334867; Carrageenan test; Spinal cord

1. Introduction

Orexin-A and orexin-B are hypothalamic peptides of 33 and 28 amino acids, respectively, and have a role in the regulation of feeding behavior, energy metabolism, and the sleep–wake cycle. Orexin-A and orexin-B act via G-protein coupled receptors named the orexin-1 receptor and the orexin-2 receptor (Sakurai et al., 1998). Orexin-A binds equally to both orexin-1 and orexin-2 receptors, while orexin-B has a preferential affinity for orexin-2 receptors (Sakurai et al., 1998).

Carrageenan injection into the rat hindpaw induces mechanical allodynia and thermal hyperalgesia, and these mechanical allodynia and thermal hyperalgesia have been used as a model of inflammatory pain (the carrageenan test) (Yamamoto et al., 1993, 2001). Intravenous administration of orexin-A has been reported to attenuate the level of thermal hyperalgesia in the mouse carrageenan test and this effect of orexin-A has been reported to be mediated by the activation of peripheral orexin-1 receptor (Bingham et al., 2001). Moreover, intracerebroventricular injection of orexin-A produces an analgesic effect in the rat hot plate test (Bingham et al., 2001). These data may suggest that peripheral and supraspinal orexinergic systems have a potential role in the modulation of nociceptive transmission.

Van den Pol (1999) demonstrated the presence of robust projection of orexin-B from the hypothalamus to lamina I of the spinal cord. Moreover, Date et al. (2000) reported that both orexin-A and orexin-B were distributed throughout the spinal cord and that, in the spinal cord, orexin fibers were concentrated in lamina I of the dorsal horn and in lamina X surrounding the central canal. Hervieu et al. (2001) reported that orexin-1 receptor is localized on C-fibers in the spinal cord. These data suggest that spinal orexinergic system is involved in the nociceptive information transmission. Moreover, the authors have been reported that the activation of spinal orexin-1 receptor produces an analgesic effect during the rat formalin test (Yamamoto et al., 2002). Although both the formalin test and the carrageenan test have been used as models of inflammatory pain, there are several differences between the two tests. Paw formalin injection induces biphasic spontaneous nociceptive behavior, such as flinching, with a duration of about 1 h. On the other hand, paw carrageenan injection induces no flinching response, and induces thermal hyperalgesia and mechanical allodynia and

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much more severe paw edema. Thus, it is possible that the role of spinal orexin-1 receptor in the formalin test is different from that in the carrageenan test. In the present study, to define the role of orexin-1 receptor in nociceptive transmission in the spinal cord during the carrageenan test, the authors examined the effect of intrathecal administration of orexin-A on the carrageenan test in the rat.

2. Methods

The following investigations were performed according to a protocol approved by the Institutional Animal Care Committee of Chiba University, Chiba, Japan. Male Sprague–Dawley rats weighing 250–300 g were fitted with long-term intrathecal catheters and observed for the effect of the drug.

2.1. Intrathecal catheters

Chronic intrathecal catheters were inserted, during halothane anesthesia, by passing a PE-10 catheter through an incision in the atlanto-occipital membrane to a position 8 cm caudal to the cisterna at the level of lumbar enlargement (Yaksh and Rudy, 1976). The catheter was externalized on the top of the skull and sealed with a steel wire, and the wound was closed with 3–0 silk sutures. The animals were allowed to recover for 1 week before being used experimentally. All animals displayed normal feeding and drinking behavior post-operatively. Rats showing neurological deficits were not studied.

2.2. Carrageenan model

Two milligrams of lambda carrageenan (Sigma, St. Louis, MO) was injected subcutaneously via a 24-gauge needle into the plantar surface of the right hind paw under halothane anesthesia. The lambda carrageenan was suspended in normal saline by sonication and was administered in a 0.1-ml injection volume.

2.3. Assessments of mechanical allodynia and thermal hyperalgesia

2.3.1. Mechanical allodynia

Mechanical thresholds were measured using von Frey filaments with logarithmically incremental stiffnesses (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50 and 15.1 g) (Stoelting, Wood Dale, IL, USA) to calculate the 50% probability thresholds for mechanical paw withdrawal, as previously described (Chaplan et al., 1994). Withdrawal thresholds (50%) were calculated according to the method of Dixson (1980). For the measurement of mechanical threshold, rats were placed in a plastic cage with a wire mesh bottom. Beginning with the 2.00-g probe, filaments were applied to the plantar surface of a hind paw for 6–8 s. Stimuli were always presented in a consecutive fashion, whether ascending or descending. In the absence of a paw withdrawal response to the initial selected filament, a stronger stimulus was presented; in case of paw withdrawal, the next weaker stimulus was chosen. Stimuli were presented at intervals of several seconds, allowing for apparent resolution of any behavioral responses to previous stimuli. According to Dixson (1980), optimal threshold calculation by this method requires six responses in the immediate vicinity of the 50% threshold. Although all responses were noted, counting of the critical 6-data points did not begin until the response threshold was first crossed, at which time the two responses straddling the threshold were retrospectively designated as the first two responses of the series of 6. 50% withdrawal threshold was interpolated using the formula:

$$50\%\ \text{threshold}\ (\text{g}) = (10^{[\text{Xf}+\kappa\delta]})/10,000$$

where Xf = value (in log units) of the final von Frey filament used; κ = tabular value (see Chaplan et al., 1994) for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli (here, 0.224). In case where continuous positive or negative responses were observed to the exhaustion of the stimulus set, values of 15.00 and 0.25 g were assigned, respectively.

2.3.2. Thermal hyperalgesia

Paw withdrawal latency in response to thermal stimulation was measured with a device similar to that previously reported (Hargreaves et al., 1988). The rats were placed in 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-12 V-100 W, 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 the glass temperature was regulated at 30 °C. The calibration of the thermal test system was such that the average response latency in 10 normal untreated rats was maintained at 10 s before the initiation of an experimental 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. Care was taken not to focus the lamp on skin not in contact with the glass floor. 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 time value was then assigned as the response latency. The trial was terminated and the lamp removed in the absence of a response within 20 s.

2.4. Behavioral analysis

The general behavior of each rat was carefully observed and tested. Motor functions were evaluated by the performance of two specific behavioral tasks, as follows. (1) The placing/stepping reflex: this response was evoked by drawing the dorsum of either hindpaw over the edge of a tabletop. In normal animals, this stimulus elicits an upward lifting of the paw onto the surface of the table, called stepping. Animals with any degree of hind limb flaccidity will demonstrate an altered or absent reflex. (2) The righting reflex: an animal placed horizontally with its back on the table will normally show an immediate coordinated twisting of the body around its longitudinal axis to regain its normal position on its feet. Animals displaying ataxic behavior will show a decreased ability to right themselves. To quantify the evaluation of motor functions, both tasks were scored on a scale of 0 to 2 in which 0 = absence of function and 2 = normal motor functions. Animals that were able to perform the motor tasks but did so more slowly than normal animals were assigned a score of 1.

2.5. Drugs

The intrathecally administered drugs were delivered in a total volume of 10 μl followed by 10 μl of saline to flush the catheter. The agents used in this study were orexin-A (molecular weight = 3561, Peptide Institute, Osaka, Japan) and SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride, molecular weight = 356, GlaxoSmithKline, Herts, UK), a selective orexin-1 receptor antagonist (Porter et al., 2001; Smart et al., 2001).

2.6. Experimental protocol

A preliminary study performed in the authors' laboratory revealed that the maximum level of mechanical allodynia and thermal hyperalgesia occurred 2 h after the carrageenan injection and lasted for more than 4 h after the development of mechanical allodynia and thermal hyperalgesia. Thus, drugs were administered 3 h after the carrageenan injection. Before and 3 h after the carrageenan subcutaneous injection, the mechanical threshold or paw withdrawal latency against thermal stimulation of the right hind paw was measured as control data. Then drugs were administered intrathecally, and the right hind paw was tested at 5, 15, 30, 60 and 90 min after the drug administration. To obtain control data, vehicle was injected intrathecally. In separate groups of rats, to verify whether the effect of intrathecally administered orexin-A on mechanical allodynia in the carrageenan model was mediated by the activation of spinal orexin-1 receptor, 30 nmol of SB-334867 was administered intrathecally 5 min before the intrathecal injection of 0.3 nmol of orexin-A and the effect on the carrageenan model was examined (antagonist study). The effect of intrathecal administration of 30 nmol of SB-334867 on the carrageenan test was also examined. After the experiment, the animals were killed with an overdose of barbiturate.

2.7. Statistical analysis

To determine whether carrageenan injection induced significant mechanical allodynia or thermal hyperalgesia, we compared the pre-injection mechanical threshold or paw withdrawal latency with the pre-drug mechanical threshold or paw withdrawal latency, respectively, with a *t*-test. To analyze the effect of drugs on the mechanical threshold or paw withdrawal latency, the area under the curve (AUC) was calculated by use of the trapezoidal rule over the entire time course of the time–effect curve. For the dose–response analysis, the AUC above the pre-drug level (AUC_{above} = AUC − (pre-drug mechanical threshold (g) or pre-drug paw withdrawal latency (s) × 90 min)) was used. The AUC_{above} indicated the amount of the increase in AUC after the drug administration, regardless of the level of the pre-drug value. To analyze the dose-dependency, one-way analysis of variance (ANOVA) with Dunnetts multiple comparison test was used. In the antagonist study, a *t*-test was used.

Whenever appropriate, results are expressed as mean ± S.D. Critical values that reached a $P < 0.05$ level of significance were considered significant.

3. Results

3.1. Behavioral analysis

After the intrathecal injection of orexin-A or SB-334867, all animals scored 2 (normal motor function) in the placing/stepping reflex and righting reflex tests.

3.2. Mechanical allodynia

The carrageenan injection significantly decreased the mechanical threshold 3 h after the carrageenan injection as compared with the pre-carrageenan mechanical threshold (pre-carrageenan = 14.9 ± 0.3 g, 3 h after carrageenan = 2.0 ± 1.1 g ($n = 38$), $P < 0.001$ by *t*-test), and significant mechanical allodynia occurred 3 h after the carrageenan injection.

No difference was apparent between pre-drug mechanical thresholds in each group [3 nmol of orexin-A-treated group ($n = 5$): 2.1 ± 0.8 g; 0.3 nmol of orexin-A-treated group ($n = 5$): 1.9 ± 1.4 g; 0.03 nmol of orexin-A-treated group ($n = 5$): 1.8 ± 1.4 g; 0.003 nmol of orexin-A-treated group ($n = 6$): 2.8 ± 1.5 g; saline-treated group ($n = 7$): 2.1 ± 1.1 g; SB-334867 30 nmol + orexin A 0.3 nmol-treated group ($n = 5$): 1.6 ± 0.1 g; SB-334867 30 nmol-treated group ($n = 5$): 1.7 ± 0.4 g, $P > 0.5$ by ANOVA].

Intrathecal injection of 0.3 nmol of orexin-A significantly increased the level of AUC_{above} as compared with the saline-

treated rats and intrathecal injection of 3 nmol of orexin-A showed a plateau effect ($P<0.05$ by ANOVA; Figs. 1 and 2). In the antagonist study, 30 nmol of SB-334867 had no effect on the level of AUC_{above} as compared with the saline-treated rats (SB-334867-treated rats: 1.15 ± 55.0; saline-treated rats: -5.94 ± 59.0, Fig. 3, $P>0.8$ by t-test). Pre-treatment with 30 nmol of SB-334867 antagonized the anti-allodynic effect of 0.3 nmol of orexin-A (SB-334867 + orexin-A-treated rats: 39.3 ± 57.4; orexin-A-treated rats: 184 ± 103, Fig. 3, $P<0.05$ by t-test).

3.3. Thermal hyperalgesia

The carrageenan injection significantly decreased the level of the paw withdrawal latency 3 h after the carrageenan injection as compared with pre-carrageenan paw withdrawal latency (pre-carrageenan = 11.0 ± 1.1 s, 3 h after carrageenan = 5.5 ± 1.3 s ($n=23$), $P<0.001$ by t-test), and significant thermal hyperalgesia occurred 3 h after the carrageenan injection.

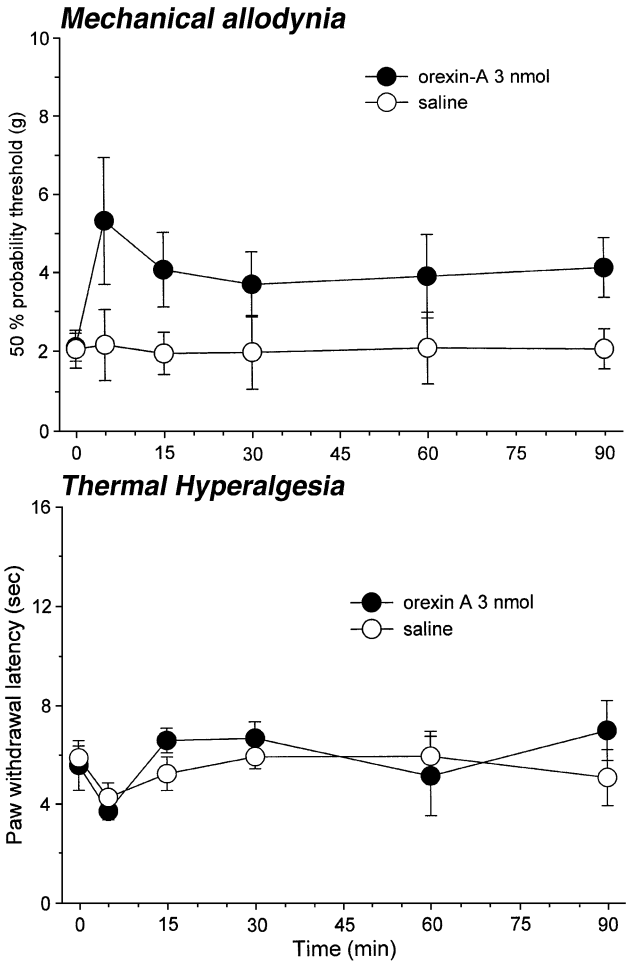

Fig. 1. Effects of intrathecal injection of 3 nmol of orexin-A and saline on the time courses of the mechanical threshold (top) and paw withdrawal latency against thermal stimulation (bottom) in the carrageenan model. Mechanical threshold (g) or paw withdrawal latency (s) was measured in the injected paw. Drugs were administered intrathecally 3 h after the paw carrageenan injection. Top: Ordinate = 50% probability threshold for mechanical paw withdrawal [50% threshold (g)]; abscissa = time after the drug administration (min). Bottom: Ordinate = paw withdrawal latency against thermal simulation (s); abscissa = time after the drug administration (min). Each line represents the group mean and S.E.M. of four to seven rats.

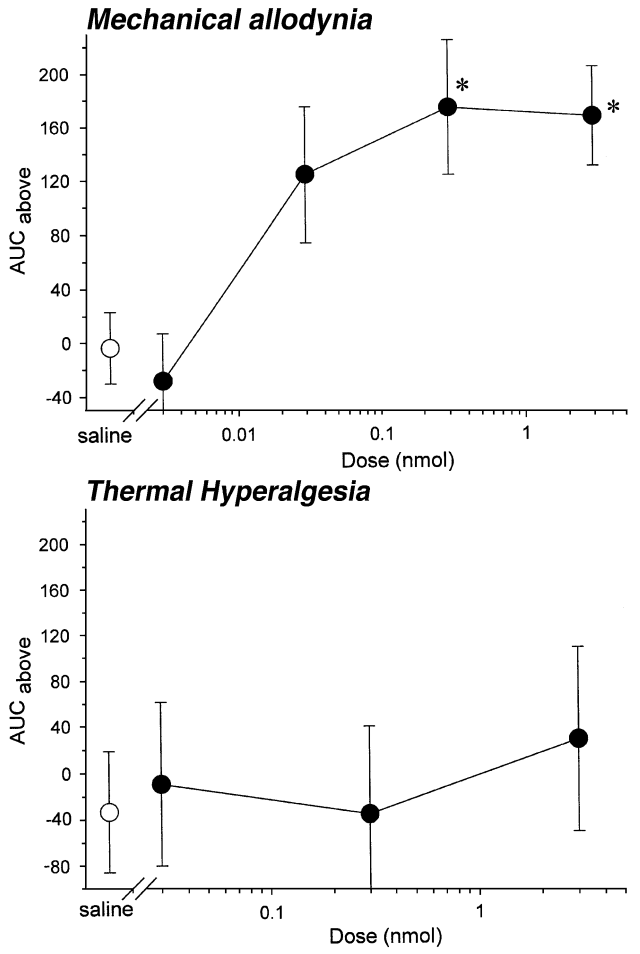

Fig. 2. Log dose–response curves for the effect of orexin-A on the AUC above the pre-drug level (AUC_{above}) of the time effect curve of orexin-A in mechanical allodynia (top) and thermal hyperalgesia (bottom). Drugs were administered intrathecally 3 h after the carrageenan injection. Each point represents the mean ± S.E.M. determination made in four to seven rats. In addition, 0.3 and 3 nmol of orexin-A increased the level of AUC_{above} in mechanical allodynia, but not in thermal hyperalgesia, as compared with saline-treated rats. *$p<0.05$ as compared with the saline-treated rats. The abscissa shows the log dose (nmol), and the ordinate shows the AUC_{above} of the time–effect curve.

No difference was apparent between pre-drug thermal withdrawal latency in each group (3 nmol of orexin-A-treated group ($n=4$): 5.6 ± 2.0 s; 0.3 nmol of orexin-A-treated group ($n=5$): 5.0 ± 1.3 s; 0.03 nmol of orexin-A-treated group ($n=4$): 5.5 ± 1.2 s; saline-treated group ($n=5$): 5.9 ± 1.1 s; SB-334867 30 nmol-treated group ($n=5$): 5.6 ± 1.5 s, $p>0.8$ by ANOVA).

Intrathecal injection of orexin-A had no effect on the level of AUC_{above} as compared with the saline-treated rats at doses between 0.03 and 3 nmol ($P>0.9$ by ANOVA; Figs. 1 and 2). In the antagonist study, 30 nmol of SB-334867 had

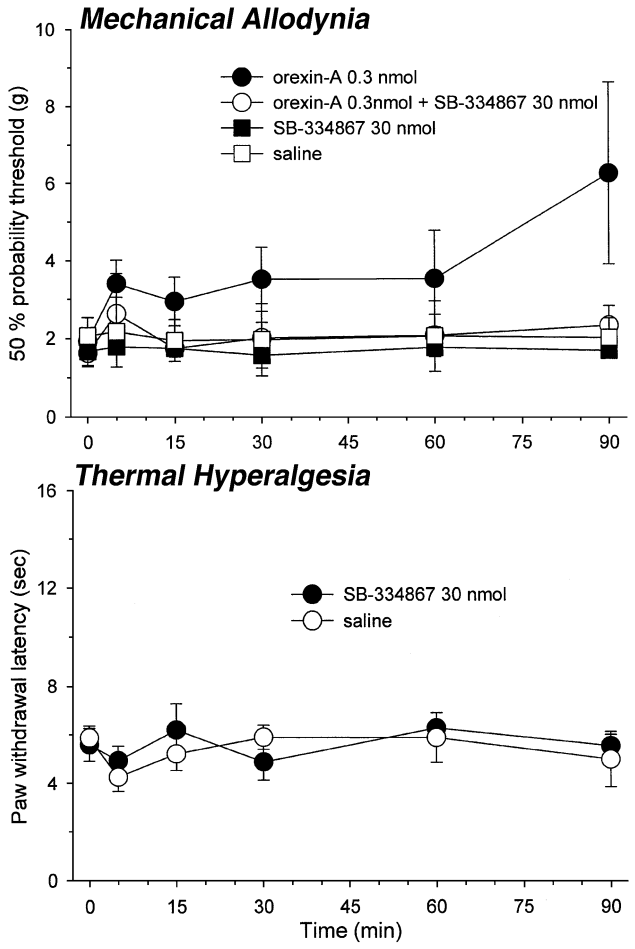

Fig. 3. Effects of intrathecal injection of 0.3 nmol of orexin-A + 30 nmol of SB-334867 and 30 nmol of SB-334867 on the time course of the mechanical threshold (top) and effects of intrathecal injection of 30 nmol of SB-334867 on the time course of the paw withdrawal latency against thermal stimulation (bottom) in the carrageenan model. Mechanical threshold (g) or paw withdrawal latency (s) was measured in the injured paw. Drugs were administered intrathecally 3 h after the paw carrageenan injection. Top: Ordinate = 50% probability threshold for mechanical paw withdrawal [50% probability threshold (g)]; abscissa = time after the drug administration (min). Bottom: Ordinate = paw withdrawal latency against thermal simulation (s); abscissa = time after the drug administration (min). Each line represents the group mean and S.E.M. of five rats.

no effect on the level of AUC_{above} as compared with the saline-treated rats (SB-334867-treated rats: 5.85 ± 164; saline-treated rats: -33.5 ± 117, Fig. 3, $P>0.6$ by t-test).

4. Discussion

In the present study, the authors have clearly demonstrated that intrathecal injection of 0.3 nmol of orexin-A attenuated the level of mechanical allodynia induced by paw carrageenan injection and 3 nmol of orexin-A produced a plateau effect. Intrathecal injection of 30 nmol of SB-334867 had no effect on the level of mechanical allodynia and pre-treatment with 30 nmol of SB-334867 completely antagonized the anti-mechanical allodynic effect of 0.3 nmol of orexin-A. This suggests that the activation of spinal orexin-1 receptors produced an anti-mechanical allodynic effect in the rat carrageenan test. It is possible that intrathecally administered orexin-A activated the peripheral orexin-1 receptors and produced the anti-mechanical allodynic effect. Bingham et al. (2001) reported that intravenous injection of 3 and 9 μmol/kg of orexin-A reduces the level of thermal hyperalgesia in the mouse carrageenan test. In the present study, spinally applied 0.3 and 3 nmol/rat of orexin-A produced an anti-mechanical allodynic effect and these doses are much smaller than intravenous doses. This also suggested that, in the present study, intrathecally applied orexin-A acted at the spinal cord and that an anti-mechanical allodynic effect of intrathecally administered orexin-A is mediated by the activation of spinal orexin-1 receptors.

In the present study, the authors measured the mechanical threshold and the thermal withdrawal latency of the ipsilateral hindpaw. It has been reported that, in the rat carrageenan test, the level of mechanical threshold is highly dependent of the weight bearing of the hind limb (Kauppila et al., 1998). In the carrageenan test, the animal usually supports its weight using the contralateral hind limb and the carrageenan-injected hind limb is not weight bearing. Thus, authors think that it is not meaningful to compare the level of mechanical threshold of the carrageenan-injected hind limb with that of the contralateral hind limb.

Intrathecal administration of orexin-A had no effect on thermal hyperalgesia induced by paw carrageenan injection at doses which suppressed the level of mechanical allodynia in the present study. It is possible that higher doses of orexin-A may suppress the level of thermal hyperalgesia but our data strongly suggest that, in the spinal cord, the mechanisms of thermal information transmission are different from those of mechanical information transmission during the rat carrageenan test. It has been reported that, in the partial sciatic nerve ligation model, mechanical allodynia is mediated by A-fibers and that thermal hyperalgesia is mediated by C-fibers (Shir and Seltzer, 1990) and this data suggested that stimulus specific nociceptive transmission system may exist. The authors think that this is why mechanical allodynia is spinal orexin-1 receptor-dependent pain and thermal hyperalgesia is not spinal orexin-1 receptor-dependent pain.

As mentioned in Introduction, it has been reported that intravenous injection of orexin-A reduces the level of thermal hyperalgesia induced by paw carrageenan injection and this effect of orexin-A is mediated by the activation of peripheral orexin-1 receptor (Bingham et al., 2001). Thus, the role of spinal orexin-1 receptor in the transmission of thermal nociceptive information is different from that of peripheral orexin-1 receptor during carrageenan test.

It has been reported that intravenous administration of SB-334867 produces a pro-hyperalgesic effect in the paw carrageenan induced thermal hyperalgesia test (Bingham et al., 2001). In the present study, intrathecal injection of SB-

334867 had no effect on the level of thermal hyperalgesia, or on the level of mechanical allodynia, at a dose that antagonized the effect of 0.3 nmol of orexin-A on the mechanical allodynia induced by paw carrageenan injection. These data suggest that, during the carrageenan test, the peripheral orexin-1 receptors are tonically activated but the spinal orexin-1 receptors are not tonically activated.

The precise mechanism by which intrathecally administered orexin-A produces an anti-allodynic effect during the carrageenan test is unclear. The authors recently found that intrathecally administered orexin-A produced an analgesic effect in the rat formalin test and that it suppressed the expression of Fos-like immunoreactivity induced by paw formalin injection in laminae I–II of the spinal cord (Yamamoto et al., 2002). These data suggest that orexin-A suppresses the nociceptive input to laminae I–II of the spinal cord during the formalin test. It is possible that, during the carrageenan test, spinally applied orexin-A also suppressed the nociceptive input to laminae I–II of the spinal cord and produced an anti-mechanical allodynic effect.

In conclusion, intrathecal injection of orexin-A activated the spinal orexin-1 receptors and attenuated the level of mechanical allodynia, but not thermal hyperalgesia, induced by paw carrageenan injection. Spinal orexin-1 receptors are not tonically activated during carrageenan test. These data suggest that the spinal orexin-1 receptor system modulates mechanical information transmission during the rat carrageenan test.

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