Antiallodynic Effects of Intrathecal Orexins in a Rat Model of Postoperative Pain

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ABSTRACT

Orexin A and B (hypocretin 1 and 2) are the endogenous ligands of orexin receptors, a G-protein-coupled orphan receptor family containing orexin 1 (OX1) and orexin 2 (OX2) types. Orexin A induces analgesia in acute and inflammatory pain models. We further elucidated the possible antiallodynic effect of intrathecal orexins in a rat model of postoperative pain. Mechanical allodynia was induced by incising the rat hind paw and evaluated with the withdrawal threshold to von Frey filament stimulation. Intrathecal orexin A (0.03–1 nmol) and orexin B (0.1–3 nmol) dose dependently attenuated the incision-induced allodynia. Orexin A (ED50 = 0.06 nmol) is more potent than orexin B. The effects of orexin A and B were abolished by their respective antibodies, but not by naloxone, and were attenuated by suramin and strychnine, the P2X purinergic and glycine receptor antagonists, respectively. SB-334867, an OX1 receptor antagonist, at 30 nmol completely blocked the effect of orexin A but, even at 100 nmol, only partially antagonized the effect of orexin B. Orexin A antibody, SB-334867, suramin, strychnine, or naloxone enhanced the incision-induced allodynic response. It is concluded that intrathecal orexins reduce incision-induced allodynia through OX1 receptors. Glycine and P2X purinergic receptors, but not opioid receptors, might be involved in the antiallodynic effects of orexins. Endogenous orexin might be released after incision injury to activate the spinal OX1 receptors as an endogenous analgesic protector.

Orexin A and B, also known as hypocretin 1 and 2, are peptides of 33 and 28 amino acids, respectively, derived from a single precursor, prepro-orexin, which is exclusively expressed in neurons of the lateral and posterior hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). Orexin-containing neurons send axonal projections widely throughout the entire nervous system (Peyron et al., 1998; Chen et al., 1999; Date et al., 1999; van den Pol, 1999). Two types of orexin receptors, orexin 1 (OX1) and orexin 2 (OX2), have been identified from G-protein-coupled orphan receptors (Sakurai et al., 1998). OX1 receptors exhibit high affinity for orexin A, whereas OX2 receptors display equal affinity for both orexin A and B (Sakurai et al., 1998). The distribution pattern of OX1 and OX2 receptors is generally consistent with that of the orexin-containing processes in the brain (Sakurai et al., 1998), although a differential distribution of the two receptors is notable (Marcus et al., 2001).

Orexins have been implicated in several hypothalamic functions such as the regulation of appetite (Sakurai et al., 1998; Yamamoto et al., 1999), drinking (Kunii et al., 1999), and neuroendocrine functions (Date et al., 1999; Russell et al., 2001) as well as in arousal (Chemelli et al., 1999; Lin et al., 1999) and central control of autonomic activity (Date et al., 1999; Chen et al., 2000; Hwang et al., 2001). In addition, several lines of evidence imply orexins are involved in nociceptive sensory processes. Orexin-producing neurons in the hypothalamus express the opioid peptide dynorphin (Risold et al., 1999). OX1 and OX2 receptor mRNA are moderately expressed in the midbrain periaqueductal gray (Marcus et al., 2001), a crucial pain processing area, in which the expression of Fos was strongly stimulated following an intracerebroventricular injection of orexins (Date et al., 1999). In a behavior study, Bingham et al. (2001) found that orexin A, when administered intravenously and intracerebroventricularly but not subcutaneously, possessed analgesic effects in carrageenan-induced thermal hyperalgesia, hot-plate, and abdominal constriction tests in mice.

In the spinal cord, orexin-containing fibers are densely distributed in superficial dorsal horn neurons that are important for nociceptive transmission (van den Pol, 1999;
Bingham et al., 2001). The electrophysiological study of Grudt et al. (2002) also shows that orexin B activated certain superficial dorsal horn neurons located in the lamina II of spinal cord slices. Orexins may also act at the spinal cord to regulate nociceptive transmission. Therefore, the present study was designed to elucidate whether intrathecal injection of orexins would induce allodynia in a postoperative pain model developed by Brennan et al. (1996), in which mechanical allodynia could be induced by paw incision in the rat and lasting for 2 to 3 days, mimicking the time course of clinical postoperative pain (Zahn et al., 1997). The findings of Grudt et al. (2002) show that the excitatory effect of orexin B is partially mediated through \( P_{2X} \) purinergic receptors and that orexin B enhances inhibitory, exclusively the glycineergic but not the GABAergic, synaptic transmission in the majority of recorded neurons. Therefore, the possible involvement of \( P_{2X} \) purinergic and glycine receptors in the antiallodynic effects of orexins was also investigated in this study.

### Materials and Methods

This study was approved by the Animal Care and Use Committee of the College of Medicine, National Taiwan University. Intrathecal catheterization, postoperative allodynia induction, and analysis of allodynic responses were similar to our previous report (Cheng et al., 2003).

**Intrathecal Catheterization.** For intrathecal drug administration, intrathecal catheterization (LoPachin et al., 1981) was performed by advancing the PE-5 catheter 8.5 cm caudally to the lumbar enlargement through an incision in the cisternal membrane of male Wistar rats (230–270 g) that were under isoflurane mask anesthesia. The catheter was externalized and secured to the musculature at the incision site. Rats showing any neurologic dysfunction, such as paralysis or urine incontinence, after catheterization were euthanized. A proper location of the catheter was confirmed by the hind limb paralysis after intrathecal injection of lidocaine (2%, 10 \( \mu l \) 1 day after catheterization. Only rats displaying normal grooming, ambulation, and weight gain after catheterization were used in the following study.

**Postoperative Allodynia Induction.** Postoperative allodynia was induced 5 days after intrathecal catheterization according to the method described by Brennan et al. (1996). Under isoflurane mask anesthesia, rats were subjected to an 1-cm longitudinal incision over the plantar surface of the right hind paw, and the plantaris muscle was incised longitudinally. The incision was closed with two mattress stitches, and the rats were then allowed to recover from general anesthesia.

**Von Frey Filament Testing.** The allodynic responses were evaluated by the withdrawal thresholds to punctate mechanical stimuli induced by calibrated von Frey filaments with logarithmically incremental stiffness from 0.6 to 26 g (Stoelting Co., Wood Dale, IL). Beginning with the 4-g probe, the filament was applied vertically to the area adjacent to the incision wound for 6 s while the filament was bent. Brisk withdrawal or paw flinching was considered as a positive response. If a positive response was observed, the filament with the next lower force was applied; otherwise, the next stiffer filament was used. The stimulus producing a 50% likelihood withdrawal was determined using the Dixon “up-down” method, as described by Chaplan et al. (1994). Rats with postincision withdrawal thresholds less than 5 g were considered to have marked allodynia and were used in the present study. The withdrawal thresholds were measured twice (with 2 min in between) and averaged before and after paw incision and every 5 to 30 min for 3 h after intrathecal treatments.

**Intrathecal Treatments.** Two hours after paw incision, the postincision withdrawal threshold was determined followed by intrathecal treatments. Orexin A or B was injected intrathecally preceded by normal saline, solvent control, or various antidotes, including the respective antibodies of orexin A and B and the selective antagonists of \( OX_1 \), opioid, \( P_{2X} \) purinergic and glycine receptors, respectively, SB-334867, naloxone, suramin and strychnine. All the agents were dissolved in normal saline, unless stated otherwise, and injected intrathecally in a volume of 5 \( \mu l \) followed by a 10-\( \mu l \) normal saline flush. Rats showing motor dysfunction, including abnormal ambulation and placing/stepping reflex, after intrathecal treatments were excluded from the study.

**Chemicals.** Orexin A and B were purchased from Phoenix Pharmaceuticals (Belmont, CA), and their antibodies were from Oncogene Research Products (Cambridge, MA). SB-334867 hydrochloride was a generous gift from GlaxoSmithKline (Harlow, UK). Naloxone hydrochloride and strychnine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO), and suramin hexaamid was from Tocris Cookson Inc. (Bristol, UK).

### Data Analysis and Statistics

The antiallodynic effect of orexins was evaluated by the increment of the withdrawal threshold after the intrathecal treatment and expressed as percentage of maximal possible effect (%MPE):

\[
\%MPE = \frac{\text{withdrawal threshold}_{\text{post-treatment}} - \text{withdrawal threshold}_{\text{preincision}}}{\text{withdrawal threshold}_{\text{postincision}} - \text{withdrawal threshold}_{\text{preincision}}} \times 100
\]

The withdrawal thresholds after the treatments of antidotes alone were also monitored. In animals with postinjection withdrawal threshold higher than the lowest limit (0.6 g) of the von Frey filament stimuli, it is possible to obtain a negative value of %MPE if the withdrawal thresholds were decreased after treatments, i.e., to enhance the antiallodynic response.

Data are presented as mean ± S.E.M. with \( n \) indicating the number of rats tested in each group. The time course in each treatment group was constructed by averaging the responses of the rats in the group at the same time point, and the differences between groups were compared with two-way analysis of variance (ANOVA) with post hoc Dunnett’s test. The peak antiallodynic effect from individual rats in the same group was pooled and averaged, and the differences between groups were compared with Student’s t test.

### Results

From 302 rats tested in total, the averaged withdrawal threshold before paw incision is 14.0 ± 0.3 g and that after incision is 1.16 ± 0.03 g.

**Antiallodynic Effects of Intrathecal Orexin A and B.** Intrathecal injection of orexin A (0.03–1 nmol) dose dependently reduced the mechanical allodynia induced by paw incision in the rat. The antiallodynic effect of orexin A peaked at 20 to 30 min after injection and lasted for more than 2 h (Fig. 1A). Intrathecal administration of orexin B, at 0.1 to 3 nmol, also produced antiallodynia dose dependently with the time course similar to that of orexin A (Fig. 1B). Motor weakness was observed when 10 nmol of orexin B was injected.

The peak antiallodynic effects induced by various doses of orexin A and B were shown in Fig. 2. The ED\(_{50}\) of orexin A estimated from the dose-response curve is 0.059 ± 0.003 nmol. The maximal antiallodynic effect of orexin B was unavailable owing to its motor weakening effect at 10 nmol. It is, therefore, hard to predict the ED\(_{50}\) of orexin B from the logistic fitting. Nevertheless, the dose-response curves show that orexin B appears to be much less potent than orexin A (Fig. 2).
orexin B was not available since it produced motor weakness at 10 nmol.

Effects of Orexin Antibodies on the Antiallodynic Effects of Orexins. The antiallodynic effect of orexin A, 0.3 nmol, was markedly antagonized by the polyclonal antibody (1:500 dilution) specific to orexin A (Fig. 3, A and C). Similarly, the effect of orexin B (0.3 nmol) was blocked by the purified IgG antibody raised against orexin B, at doses higher than 0.05 μg (Fig. 3, B and D). Interestingly, when the orexin A antibody was given alone, the %MPE value obtained was negative (Fig. 3, A and C). This suggests that orexin A antibody increases incision-induced allodynic response. Similarly, the orexin B antibody (0.05–0.1 μg) also slightly, though not statistically significantly, increased the incision-induced allodynia (Fig. 3, B and D).

Effects of SB-334867 on the Antiallodynic Effects of Orexins. To elucidate whether the antiallodynic effects of orexins are mediated through orexin receptors, SB-334867, a selective OX1 receptor antagonist, was administered intrathecally followed by orexins. The antiallodynic effect of orexin A at 0.3 nmol was completely abolished by 30 nmol of SB-334867 (Fig. 4, A and C). On the other hand, SB-334867, at 30 nmol, only partially antagonized the antiallodynic effect of orexin B (Fig. 4, B and D). Elevating the dose of SB-334867 to 100 nmol by dissolving it in pure dimethylsulfoxide, which did not affect the antiallodynia induced by orexins, further decreased, though not completely block, the effect of orexin B (Fig. 4, B and D). Similar to the effects of orexin antibodies alone, 30 or 100 nmol of SB-334867 significantly enhanced the incision-induced allodynia (Fig. 4, A and C).

Effect of Naloxone on the Antiallodynic Effects of Orexins. The opioid receptor antagonist, naloxone, was intrathecally administered followed by orexins to evaluate whether opioid receptors are involved in the antiallodynic effects of orexins. Naloxone (50 nmol) did not affect the antiallodynia induced by 0.3 nmol of orexin A (peak effect: 31.1 ± 3.9 %MPE, n = 8 versus 26.4 ± 5.7 %MPE, n = 12) or orexin B (peak effect: 15.4 ± 2.4 %MPE, n = 7 versus 15.1 ± 2.5 %MPE, n = 10). However, when 50 nmol of naloxone was intrathecally injected alone, the incision-induced allodynic response was significantly enhanced (peak effect: −7.6 ± 3.2 %MPE, n = 6 versus 0.2 ± 0.0 %MPE, n = 6, P < 0.05).

Effects of Suramin on the Antiallodynic Effects of Orexins. Grudt et al. (2002) reported that orexin B activated certain superficial spinal dorsal horn neurons and the effect was blocked by suramin, a P2X purinergic receptor antagonist. Therefore, the effect of suramin on orexin-induced antiallodynia was examined. At 30 nmol, suramin attenuated the antiallodynic effect of orexin A (Fig. 5). Increasing the dose of suramin to 90 nmol further attenuated, but still not completely abolished, the effect of orexin A (Fig. 5). On the other hand, the antiallodynic effect of orexin B at 0.3 nmol was attenuated by suramin at 30 nmol and totally abolished at 90 nmol (Fig. 5B).

When suramin was injected alone at 30 nmol, it produced slight antiallodynia 10 to 15 min after injection followed by allodynia enhancement (Fig. 5A). Increasing the dose of suramin to 90 nmol, a consistent enhancement of the allo-
strychnine, a glycine receptor antagonist, on the antiallodynic effect of orexin B was examined. The intrathecal dose of strychnine was tested from 40 μg based on the report of Sherman et al. (1997), which induced allodynia in anesthetized rats. However, in conscious rats, we found that intrathecal strychnine at 40 μg induced severe convulsions. The dose of strychnine was, therefore, decreased to 1 μg, at which no more seizures were noted. Strychnine, at 1 μg, significantly decreased the peak effect and shortened the duration of the antiallodynic effect of orexin B (0.3 nmol) but did not completely abolish it (Fig. 6). At a lower dose, 0.3 μg, strychnine still effectively depressed and shortened the antiallodynic effect of orexin B. Interestingly, strychnine (1 μg) alone also enhanced the incision-induced allodynia (Fig. 6).

Intrathecal Orexin A Induced Exophthalmos and Other Behavior Changes. In addition to antiallodynia, exophthalmos (proptosis) was also noted 3 min after 0.3 nmol of orexin A was injected intrathecally and lasted for about 2 h. Orexin B, to a lesser extent, also produced exophthalmos. Some other behavior changes, including grooming, face washing, and tooth grinding as well as reddening of ears and tails in the rats, were also noted following intrathecal injection of orexin A.

### Discussion

**Intrathecal Orexins Attenuate Incision-Induced Allodynia through OX₁ Receptors.** In the present study, we found that intrathecal injection of orexins induced antiallodynic effects in a rat model of postoperative pain, and orexin A was more potent than orexin B. The antiallodynic effect of orexin A, but only part of that of orexin B, is mediated through OX₁ receptors. Glycine and P₂X purinergic receptors, but not opioid receptors, might be involved in the antiallodynic effects of intrathecal orexins.

Bingham et al. (2001) found that orexin A injected intravenously or intracerebroventricularly displayed an analgesic effect mediated through OX₁ receptors in the hot-plate test and carrageenan-induced thermal hyperalgesia. Yamamoto et al. (2002; 2003) also reported that intrathecal injection of orexin A produced antinociceptive effects in formalin-induced inflammatory pain, hot-plate test, and sciatic nerve partial ligation-induced neuropathic pain models. We further demonstrated that, in the rat model of postoperative pain, intrathecal injection of orexin A exerted antiallodynic effect through OX₁ receptors. In this model, intrathecal orexin B, though being less potent than orexin A, also displayed an
antiallodynic effect. However, the effect of orexin B, different from that of orexin A, is partly mediated by OX₁ receptors. This finding is different from the report of Yamamoto et al. (2002) that no analgesic effect of intrathecal orexin B at doses up to 3 nmol was observed by hot-plate and formalin tests in rats. The reason for this discrepancy is not clear. It might be attributed to the differences in the administration time of orexin B or in the pain animal models (Yamamoto et al., 2002). The mechanism(s) involved in chemically induced inflammatory pain and incision-induced mechanical allodynia might be different, such as the extent of sensory neuron sensitization and the pattern of spinal excitatory amino acid release (Vandermeulen and Brennan, 2000; Hamalainen et al., 2002; Pogatzki et al., 2002; Zahn et al., 2002).
Given that the antiallodynic effect of orexin B was not completely antagonized by SB-334867 and that orexin B has higher binding affinity for OX1 than for OX2 receptors (Ammoun et al., 2003), the possibility that the effect of orexin B is also mediated by OX2 receptors remains to be elucidated. Nevertheless, it appears that the contribution of OX1 receptors to the antiallodynic effect of intrathecal orexins is greater than that of OX2 receptors. The finding that orexin A was more potent than orexin B may be attributed to the fact that orexin A displays 10- to 100-fold higher affinity for the OX1 receptors than orexin B (Ammoun et al., 2003).

**P2X Purinergic and Glycine, but not Opioid, Receptors are Involved in the Antiallodynic Effects of Orexins.** The ineffectiveness of naloxone in modulating the antiallodynic effect of intrathecal orexin A and B indicates that the opioid system is not involved in the antiallodynic effects of orexins. This is in agreement with the findings from Bingham et al. (2001) and Yamamoto et al. (2002), who used the mouse hot-plate test and rat formalin test, respectively. On the other hand, the attenuation of orexin-induced antiallodynia by suramin and strychnine suggests that P2X purinergic and glycine receptors are involved in their antiallodynic effects. These results provide in vivo evidence to support the in vitro findings from Grudt et al. (2002) in spinal cord slices that orexin B excites spinal dorsal horn neurons, mediated partly through P2X purinergic receptors, and increases glycnergic inhibitory transmission. Interestingly, we also found that the antiallodynic effect of orexin A was not completely blocked by suramin although that of orexin B appeared to be completely blocked.

**Protective Role of Endogenous Orexins in Incision-Induced Alloodynia.** The findings that intrathecal orexin A antibody or SB-334867 increased the incision-induced allodynia suggest that endogenous orexin A might be released after paw incision and then activate the OX1 receptors in the spinal cord as an antinociceptive protector. The insignificant change induced by intrathecal orexin B antibody may be due to either the amount of released endogenous orexin B is not enough to initiate antiallodynic action or its antiallodynic effect is insignificant since orexin B is not as potent as orexin A. Bingham et al. (2001) also reported that intraperitoneal injection of SB-334867 increased the carrageenan-induced thermal hyperalgesia but did not affect the hot-plate response. However, Yamamoto et al. (2002) did not find intrathecal SB-334867 increases the flinches in the formalin test. It is suggested that a significant protective role of endogenous orexin A, which might be released after paw incision or carrageenan intraplantar injection, does not exist in the formalin-induced inflammatory and acute thermal pain.

**Roles of Endogenous ATP, Glycine, or Opioids in Incision-Induced Alloodynia.** It is interesting to note that intrathecal injection of suramin or strychnine also increased the allodynic response. This might be because the endogenous orexin A released after paw incision activates P2X receptors indirectly and, in turn, enhances glycnergic inhibitory transmission (Grudt et al., 2002). Alternatively, it is possible that endogenous ATP and/or glycine are also released after paw incision injury in an orexin-independent manner. The possible protective role(s) of endogenous ATP or glycine in incision-induced allodynia remains to be elucidated. ATP has been found to enhance glycine release from nerve terminals of glycnergic interneurons in the dorsal horn of spinal cord and was implied to play a protective role against nociceptive stimulation (Rhee et al., 2000). Intrathecal injection of strychnine has been reported to induce tactile allodynia in rats (Loomis et al., 2001). However, intrathecal injection of suramin was found to inhibit nociceptive responses in formalin- (Driessen et al., 1994) and carrageenan-inflamed rats but not in neuropathic rats (Stansfield et al., 2000). Interestingly, Fukuhara et al. (2000) reported that intrathecal injection of suramin at low doses inhibited prostaglandin-induced allodynia but at higher doses induced allodynia in mice. In the current study, we also found that intrathecal suramin produced transient antiallodynia followed by allodynia enhancement at 30 nmol but only enhanced allodynia at 90 nmol (Fig. 5A).

Similarly, the finding that intrathecal injection of naloxone alone enhanced the postincision allodynia suggests that endogenous opioids are released after paw incision. Li et al. (2001) also demonstrated that subcutaneous injection of naloxone increased thermal hyperalgesia and mechanical allodynia in the current postoperative pain model.

**Clinical Significance of Orexins in Postoperative Pain Management.** The antiallodynic effects of intrathecal orexins observed in this study, being near 30 %MPE, is far less than those, which could be up to 80 to 100 %MPE,
produced by other analgesics used clinically, such as morphine or gabapentin, in the current postoperative pain model (Zahn et al., 1997; Cheng et al., 2003). It seems that there is little potential for orxins alone to be served clinically as an intrathecal treatment of postoperative pain. Besides, the excitophalamos and cardiovascular excitation (Chen et al., 2000; Antunes et al., 2001) induced by central administration of orxins might deter its clinical potential. However, it still remains to be elucidated if other related orxin receptor agonists, when available, can be used as adjuvant analgesics in the treatment of postoperative pain.

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References


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