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Anti-allodynic and anti-hyperalgesic effects of nociceptin receptor antagonist, JTC-801, in rats after spinal nerve injury and inflammation

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Abstract

The effects of nociceptin/orphanin FQ (N/OFQ) peptide receptor antagonist JTC-801 on allodynia and hyperalgesia were examined in rats in order to explore the involvement of N/OFQ system in these pathological pain states. Tactile allodynia induced by L5/L6 spinal nerve ligation was reversed by both systemic (3–30 mg/kg) and spinal (22.5 and 45 pg) JTC-801 in a dose-dependent manner. Concerning hyperalgesia induced by formalin injection into the hindpaw, JTC-801 dose-dependently suppressed the second phase, but not the first phase, of the licking behavior. Furthermore, systemic JTC-801 reduced Fos-like immunoreactivity in the dorsal horn of the spinal cord (laminae I/II). In conclusion, N/OFQ receptor antagonist JTC-801 exerted anti-allodynic and anti-hyperalgesic effects in rats, suggesting that N/OFQ system might be involved in the modulation of neuropathic pain and inflammatory hyperalgesia.

Keywords: N/OFQ (nociceptin/orphanin FQ); NOP receptor antagonist; JTC-801; Allodynia; Hyperalgesia

1. Introduction

Nociceptin/orphanin FQ (N/OFQ), is the most recently discovered opioid peptide which binds with high affinity to the opioid receptor-like 1 (ORL1) receptor (hereafter nociceptin/orphanin FQ peptide receptor; NOP receptor) (Meunier et al., 1995; Reinscheid et al., 1995). N/OFQ is distributed abundantly and extensively in the central nervous system (Ikeda et al., 1998; Neal et al., 1999; Houtani et al., 2000). A number of functional roles for N/OFQ have been proposed, such as stimulation of locomotor activity (Florin et al., 1996), anti-anxiety action (Jenck et al., 1997), stimulation of food intake (Stratford et al., 1997), and suppression of spatial learning (Sandin et al., 1997).

Numerous studies have examined the effects of exogenous N/OFQ on pain. However, whether it has pronociceptive or antinociceptive properties still remains unrevealed (reviewed in Xu et al., 1996; Grisel and Mogil, 2000; Mogil and Pasternak, 2001). Furthermore, the physiological role of endogenous N/OFQ in nociception has not been clearly elucidated, which could be clarified with the use of selective and potent antagonists of N/OFQ.

Recently, a small-molecule nonpeptidergic NOP receptor antagonist, JTC-801, was synthesized (Shinkai et al., 2000). JTC-801 exhibited around 12.5-, 129-, and 1055-fold selectivity for NOP receptor over μ-, κ-, and δ-opioid receptors, respectively (Shinkai et al., 2000). It also inhibited the binding of [^{3}H]-nociceptin to human NOP receptors (Ki: 44.5 nM) and completely antagonized the suppression by nociceptin of forskolin-induced accumulation of cAMP (IC_{50}: 2.58 μM) in HeLa cells in vitro (Yamada et al., 2002).

Mechanical allodynia and inflammatory hyperalgesia are likely to be to be mediated by different neuronal pathways (Sung et al., 1998; Ossipov et al., 1999; Miki et al., 2000; Sun et al., 2001). We investigated the effects of exogenous NOP receptor antagonist in two pathological pain models in rats; spinal nerve ligation model for neurogenic allodynia and formalin model for inflammatory hyperalgesia. Spinal nerve ligation induces persistent mechanical allodynia in

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rats (Kim and Chung, 1992; Kim et al., 1997) and formalin injection into the plantar surface of the hindpaw causes two phases of behavioral response, which are possibly mediated by separate mechanisms (Rosland et al., 1990; Tjølsen et al., 1992; Abbott et al., 1995). The immediate early gene, c-fos, is known to be rapidly and transiently expressed in excited neurons (reviewed in Morgan and Curran, 1989) and gives us anatomical information concerning neuronal activation (reviewed in Harris, 1998).

In the present study, we examined the effects of systemic and intrathecal JTC-801 on tactile allodynia evaluated with von Frey filaments in rats with spinal nerve ligation. We then examined the effects of systemic JTC-801 on formalin-induced hyperalgesia using a behavioral test and an immunohistochemical approach.

2. Material and methods

2.1. Animals

Experiments were performed in accordance with the guidelines on animal experimentation set by the ethics committee for animal use at Tokyo University. All rats were obtained from Nisseizai Inc. (Tokyo, Japan).

2.2. Drugs

JTC-801, *N*-(4-amino-2-methylquinolin-6-yl)-2-(4-ethhlphenoxymethyl) benzamide monohydrochloride (molecular weight 447.96), was a gift from the JT Central Pharmacological Research Institute (Osaka, Japan). Methyl cellulose was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Spinal nerve ligation and intrathecal catheterization

Male Sprague–Dawley rats weighing 130–160 g underwent spinal nerve ligation as previously described (Kim and Chung, 1992). Briefly, under isoflurane/oxygen anesthesia and with the aid of a dissecting microscope, the left L5 and L6 spinal nerves were isolated and tightly ligated with 7–0 silk thread. For the rats scheduled for intrathecal administration, a PE 10 tube (15 cm in length) was inserted cephaladly 1.5 cm through a small hole drilled at L4 lamina (Bahar et al., 1984). The other end of the tube was passed subdermally and secured to the back of the neck. After completion of the experiments, correct placement of the catheter was confirmed by lidocaine injection (140 μg/7 μl). The animal was excluded from the study if the legs were not paralyzed. After the surgery, animals were housed individually on soft beddings and were fed and watered ad libitum under constant temperature (23±2 °C) and a 12 h day/night cycle. The rats were left for recovery for at least 10 days and used for behavioral tests two to three times at 3–5 day intervals.

2.4. Immunohistochemistry

Rats were anesthetized with intraperitoneal pentobarbital (100 mg/kg) and transcardially perfused with 100 ml of 0.1 m sodium phosphate-buffered saline, followed by 500 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer cooled to 4 °C. The spinal cord was removed and immersed in the same fixative for 24 h at 4 °C. Tissues were stored overnight in 30% sucrose solution in 0.1 M phosphate buffer at 4 °C for cryoprotection. The L4–5 spinal cord was sectioned into 40-μm-thick sections with a cryotome (CM1800, Leica, Heidelberg, Germany) at −15 °C, and placed in phosphate-buffered solution. Sections were first incubated for 1 h in blocking solution (5% normal rabbit serum and 0.3% Triton X in phosphate-buffered saline) and then incubated overnight with goat anti-c-Fos antibody (1:5,000, Santa-Cruz Biotechnology, Santa-Cruz, CA) diluted in 1% normal rabbit serum and 0.3% Triton X in phosphate-buffered saline (buffer 1). After vigorous rinsing in buffer 1, sections were incubated for 1 h in biotinylated rabbit anti-goat immunoglobulin (1:200, Chemicon, Temecula, CA) in buffer 1. Sections were vigorously rinsed with 0.3% Triton X in 0.1 M phosphate-buffered saline (buffer 2) and then incubated for 1 h in avidin–biotin–peroxidase complex (Vectra Elite ABC, Vector Laboratories, Burlingame, CA) in buffer 2. Visualization of the reaction product was achieved by incubation for 4 min with diaminobenzidine and nickel-ammonium sulfate in the presence of hydrogen peroxide (diaminobenzidine kit, Vector Laboratories). All incubations were performed at room temperature. After staining, the sections were rinsed in water and placed on a glass slide. The sections were dehydrated, cleared in 100% xylene and coverslipped. Four most densely stained sections were selected in each rat and Fos-IL neurons were counted in the four regions of the spinal cord (lamina I/II, III/IV, V/VI, and VII–X) under the microscope.

2.5. Experiment 1: effect of JTC 801 on tactile allodynia

Development of tactile allodynia after spinal nerve ligation was confirmed by paw withdrawal threshold determined with von Frey filaments and up–down method (Chaplan et al., 1994). Briefly, rats were placed individually in transparent plastic boxes on a metal mesh floor and the paw was pressed for approximately 2 s with a series of von Frey filaments: 0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, 15.10 g (North Coast Medical, San Jose, CA). The filament was switched to a thicker one in the absence of paw withdrawal response, while switched to a thinner on in the event of paw withdrawal. Tests were started with a 2.00 g filament and the filaments were changed in a consecutive fashion. Fifty percent withdrawal threshold was determined from the series of responses (Dixon, 1980). The threshold less than 4.00 g was assumed to indicate allodynia (Chaplan et al., 1994). Rats with threshold over 4.00 g were excluded from the study. NOP receptor antagonist, JTC-801 (3, 10,

and 30 mg/kg in 0.5% methyl cellulose), or vehicle was orally administered using a stainless tube under light isoflurane anesthesia. Paw withdrawal threshold were measured every 30 min up to 180 min. In other group of rats, JTC-801 (22.5 and 45 pg/10 μl normal saline) or vehicle was intrathecally administered via the implanted catheter and paw withdrawal threshold was measured 15, 30, 60, 90, and 120 min later. These behavioral experiments were performed between 10 and 21 days after the spinal nerve ligation.

2.6. Experiment 2: effect of JTC-801 on inflammatory hyperalgesia

Male Sprague–Dawley rats weighing between 200 and 250 g were used for this experiment. Thirty minutes after oral administration of JTC-801 (3, 10, 30 mg/kg in 0.5% methyl cellulose) or vehicle in male Sprague–Dawley rats (200–250 g in weight) under light isoflurane anesthesia, 50 μl of 5% formalin was injected subcutaneously into the planter surface of the right hindpaw. Rats were then kept separately in a Plexiglas chamber and the total time spent licking the hindpaw was recorded at 5-min intervals for 60 min.

2.7. Experiment 3: effect of JTC-801 on formalin-induced c-Fos immunoreactivity

Male Sprague–Dawley rats weighing between 200 and 250 g were used for this experiment. Thirty minutes after oral JTC-801 (3 and 30 mg/kg in 0.5% methyl cellulose) or vehicle, 50 μl of 5% formalin was injected subcutaneously into the planter surface of the right hindpaw. One hour later, rats were fixed and the spinal cords were removed for immunohistochemical processing as described previously. All the samples were processed simultaneously to avoid variance of staining.

2.8. Data analysis and statistics

Values are expressed as mean±S.E.M. Paw withdrawal thresholds and time spent for licking behavior were expressed in grams and seconds, respectively. Behavioral data and c-Fos counts were analyzed by two-way analysis of variance (ANOVA), and post hoc comparisons by the Fisher's protected least significant differences. $P<0.05$ was considered statistically significant.

3. Results

3.1. JTC-801 and tactile allodynia

Fig. 1 shows the time course of anti-allodynic action of oral (A) and intrathecal (B) JTC-801. Spinal nerve ligation induced tactile allodynia as evidenced by remarkable reduction in paw withdrawal thresholds as compared with contralateral side (1.74±0.08 vs. 9.83±0.46 g for oral and 1.94±0.09 vs. 8.65±0.47 g for intrathecal administration). Mechanical allodynia was reversed by oral JTC-801 in a dose-dependent manner (Fig. 1A). Mechanical allodynia was also attenuated by intrathecal JTC-801 in a dose-dependent manner (Fig. 1B).

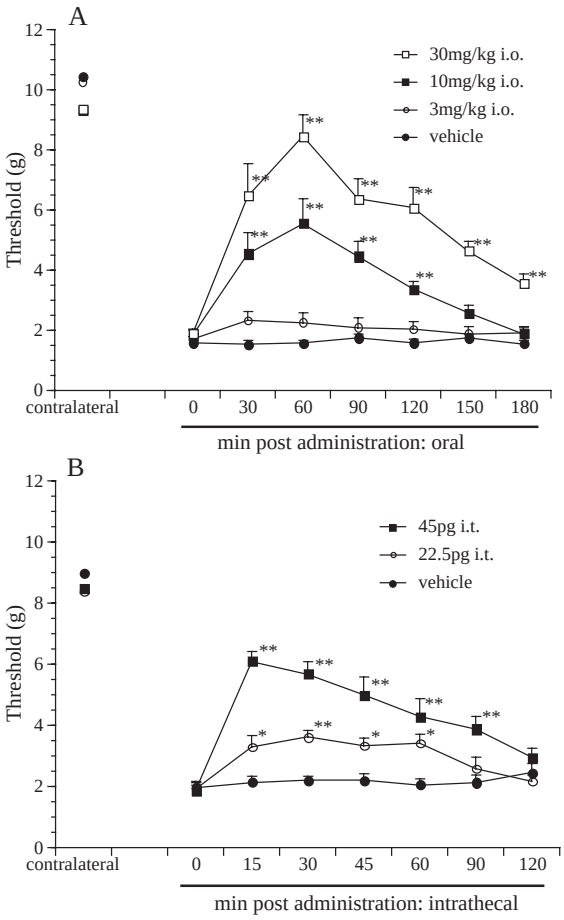

Fig. 1. Time course of anti-allodynic action of JTC-801 in rats with neuropathy produced by L5 and L6 spinal nerve ligation. A: Effect of systemic JTC-801. Rats demonstrated tactile allodynia as evidenced by significant reduction in paw withdrawal threshold when compared to the contralateral hindpaw. Graph shows data obtained in the injured paw at 30 min intervals up to 180 min after drug administration (n=8 rats per group). Tactile allodynia was reversed by oral JTC-801 in a dose dependent manner. B: Effect of intrathecal JTC-801. Graph shows data obtained in the injured paw at 15, 30, 45, 60, 90, and 120 min after drug administration (n=8 rats per group). Mechanical allodynia was reversed by intrathecal JTC-801 in a dose dependent manner. Paw withdrawal thresholds (gram) are expressed as the mean±S.E.M. *P<0.05, **P<0.01 as compared with the vehicle group.

3.2. Formalin test

Fig. 2 shows the time course of licking behavior after formalin injection in the vehicle group. Consistent with previous reports, two phases of the responses are identified; the first phase peaks between 0 and 5 min and the second phase continues between 15 and 50 min after formalin

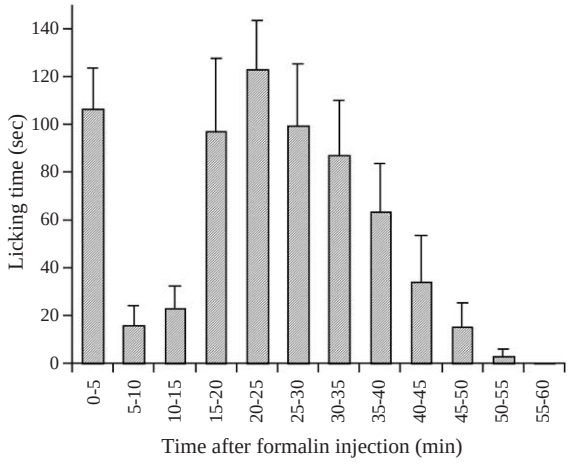

Fig. 2. Time course of licking behavior after formalin injection. There were two peaks of nociceptive behavior after intraplantar formalin; the first phase (0–5 min) with a mean licking time of 106 s, and the second phase (15–50 min) with a mean time of 520 s (n=7). Licking times (seconds) are expressed as mean±S.E.M.

injection. Oral JTC-801 pretreatment did not significantly suppress the first phase licking behavior at any doses (Fig. 3A). On the other hand, oral JTC-801 pretreatment over 3 mg/kg significantly reduced the second phase licking time (Fig. 3B). Higher dose JTC-801 (30 mg/kg) reduced total second phase licking time by 50% as compared with vehicle.

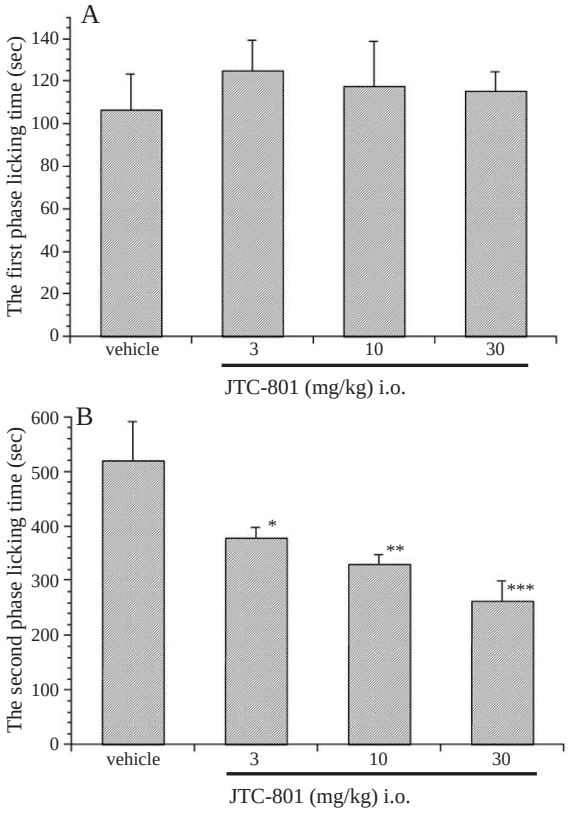

Fig. 3. Effect of JTC-801 in the rat formalin test. JTC-801 was administered intraorally 30 min before animals received 50 μl of 5% formalin into the left hind paw. JTC-801 had no effect on the first phase (0–5 min) licking time compared to vehicle group at any doses (A). JTC-801 suppressed the second phase (15–50 min) licking behavior at all doses (B). Licking times (seconds) are expressed as the mean±S.E.M. *P<0.05, **P<0.01, and ***P<0.001 as compared with the vehicle group. n=6–7 per group.

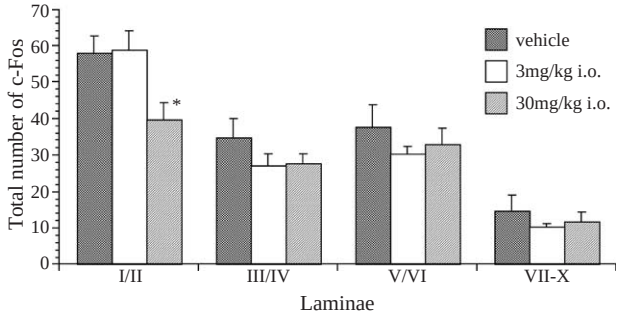

Fig. 4. Effects of JTC-801 on the formalin-induced spinal c-Fos expression. Each rat was pretreated with oral JTC-801 (3 and 30 mg/kg) or vehicle (methyl cellulose) 30 min before subcutaneous formalin injection into the hindpaw. High dose JTC-801 (30 mg/kg) suppressed the formalin-induced c-Fos expression in superficial layers (laminae I/II). In other layers, i.e. neck (laminae III/IV), dorsal (laminae V/VI), and ventral (laminae VII–X), oral JTC-801 had no effect on c-Fos expression as compared with vehicle (n=4 for each group). The number of c-Fos positive nuclei is expressed as mean±S.E.M. *P<0.05 as compared with the vehicle group.

3.3. c-Fos expression following formalin injection

The higher dose of oral JTC-801 (30 mg/kg) suppressed formalin-induced spinal c-Fos expression by 31% in the superficial layer (laminae I/II) of the spinal cord. Oral JTC-801 had no effect on c-Fos immunoreactivity in other regions of the spinal cord, i.e. neck (laminae III/IV), dorsal (laminae V/VI), and ventral (laminae VII–X) (Fig. 4). Although a few Fos-positive neurons were also observed in the contralateral side of the spinal cord, JTC-801 did not significantly reduced c-Fos positive counts in any spinal regions.

4. Discussion

JTC-801 was recently synthesized as a small-molecule nonpeptidergic NOP receptor antagonist (Shinkai et al., 2000). In the present study, we demonstrated that systemic and intrathecal JTC-801 alleviated tactile allodynia induced with spinal nerve ligation and that systemic JTC-801 inhibited formalin-induced inflammatory hyperalgesia.

Several studies have indicated that tactile allodynia with nerve injury is transmitted to the thalamus through a neuronal pathway different from that transmitting noxious stimuli by heat or chemical irritants (Miki et al., 1998, 2000; Sung et al., 1998; Sun et al., 2001). Noxious stimuli on the skin are transmitted chiefly by unmyellinated C-fibers (Lynn and Carpenter, 1982; Ossipov et al., 1999). These fibers terminate on to secondary neurons in the spinal dorsal horn connecting to the contralateral spinothalamic tract (Nieuwenhuys et al., 1981). In contrast, tactile allodynia in the

hindpaw is transmitted by large diameter myellinated Aβ fibers and the secondary fibers ascend in the spinal dorsal column up to the gracile nucleus in the medulla oblongata (dorsal column–thalamic pathway, Miki et al., 1998, 2000; Sung et al., 1998; Sun et al., 2001). Interestingly, intrathecal morphine alleviated neuropathic pain by heat stimulus (Bian et al., 1995; Mao et al., 1995), but not tactile allodynia (Bian et al., 1995; Lee et al., 1995), suggesting the involvement of different pathways. Neural plasticity in the dorsal root ganglia, dorsal horn, gracile nucleus, and ventral posterior lateral nucleus of the thalamus (VPL) may be involved in the alternation of the pathways (Noguchi et al., 1995; Miki et al., 1998; Lee et al., 1998). Because oral and intrathecal JTC-801 administration attenuated tactile allodynia in our study, we speculate that endogenous N/OFQ is involved in the pathophysiology of allodynia. Furthermore, considering the efficacy of intrathecal JTC801, spinal cord may be a major site of action of N/OFQ.

When formalin is subcutaneously injected into the hindpaw, rats show two phases of nociceptive behavior that appear to involve distinctly different physiological processes (Abbott et al., 1995). The first phase response starting immediately after injection is probably due to direct chemical activation of nociceptors and transmitted through afferent C-fibers. The second phase response starting 10–15 min after injection is likely caused by synergy of inflammation and sensitization (Tjølsen et al., 1992; Rosland et al., 1990). JTC-801 dose-dependently attenuated the second phase, but not the first phase, licking behavior in our study. Similar results were observed when some non-steroidal anti-inflammatory drugs were used; the second phase behavior was inhibited while the first phase was unaffected (Rosland et al., 1990; Carrive and Meyer-Carrive, 1997). Our results in the formalin tests might suggest that N/OFQ may be involved in neural plasticity leading to tonic hyperalgesia. Because carageenan-elicited inflammation markedly induced prepronociceptin mRNA expression, peaking within 30 min in rat dorsal root ganglion (Andoh et al., 1997), spinal nociceptin may play a part in the development of hyperalgesia.

Our results demonstrated that JTC-801 reduced formalin-induced c-Fos only in a superficial laminae (I/II). Concerning pathophysiological induction of N/OFQ by peripheral inflammation, N/OFQ levels increased in the dorsal horn but not in the ventral horn after carageenan injection into the gluteal muscle in rats (Rosen et al., 2000). N/OFQ binding increased in bilateral superficial laminae (I/II) but did not change in other laminae after complete Freund's adjuvant injection into the rat hindpaw (Jia et al., 1998). NOP are also observed in high concentrations in the superficial layers in the dorsal horn, but not in the ventral horn (Darland et al., 1998). These results might indicate that superficial layer of the spinal cord is a major site of N/OFQ action. We speculate that JTC-801 acts on NOP receptors in the superficial layers in the dorsal horn and inhibits neuronal processes leading to hyperalgesia, which may be revealed by reduced c-Fos induction in these regions.

Several mechanisms have been proposed concerning the action of NOP receptors. Mabuchi et al. reported JTC-801 diminished heat-evoked hyperalgesia in neuropathic mice by suppressing nitric oxide synthetase and decreasing nitric oxide production (Mabuchi et al., 2003). Substance P system may also be involved because substance P-induced behavioral responses (scratching, biting, and licking) were aggravated with low doses (3 amol to 3 fmol) of nociceptin, and these pronociceptive actions by substance P were mediated through NOP receptors (Inoue et al., 1999).

Several previous reports that showed that systemic and intrathecal JTC-801 had an analgesic effect on serious pain states in rodents, such as neuropathic pain (Yamada et al., 2002; Suyama et al., 2003) and inflammatory pain (Shinkai et al., 2000; Yamada et al., 2002). These results are compatible with our results that JTC-801 had anti-allodynic and anti-hyperalgesic effects. On the other hand, another non-peptidic NOP receptor antagonist, benzimidazoline J-113397, enhanced the agitation behavior when administered intrathecally and intracerebroventricularly in a rat formalin model (Yamamoto et al., 2001), suggesting an "*algesic*" action of NOP receptor antagonist. Causes of these conflicting results are still unclear and warrant further investigation. Dosage and timing of drug administration and affinities of antagonists to other neuronal receptors may be involved.

In summary, both systemic and intrathecal JTC-801 administration attenuated tactile allodynia with L5/L6 spinal nerve ligation in dose-dependent manner. JTC-801 also alleviated second phase, but not the first phase, behavioral response to formalin injection. Furthermore, JTC-801 reduced formalin-induced Fos-like immunoreactivity in laminae I/II in the ipsilateral dorsal horn. These results might suggest that nociceptin is involved in modulation of allodynia and hyperalgesia, especially at the spinal level.

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