

Full Paper

## Mechanisms of Analgesic Action of Neurotropin on Chronic Pain in Adjuvant-Induced Arthritic Rat: Roles of Descending Noradrenergic and Serotonergic Systems

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**Abstract.** Neurotropin<sup>®</sup>, a non-protein extract from the inflamed skin of rabbits inoculated with vaccinia virus, has been clinically used as an analgesic drug for treatment of chronic pain. In this study, we investigated the analgesic mechanisms of Neurotropin in the adjuvant-induced arthritic rat, a chronic pain model with inflammation. Neurotropin caused dose-dependent inhibition of hyperalgesia in the adjuvant-induced arthritic rat after single intravenous (10–100 NU/kg) and oral (30–200 NU/kg) administration. The analgesic effect of Neurotropin (intravenous 100 NU/kg and oral 200 NU/kg) was significantly inhibited by intrathecal injections of the  $\alpha_2$ -adrenoceptor antagonist yohimbine (30 nmol/animal) and the selective 5-HT<sub>3</sub> serotonin receptor antagonist MDL72222 (30 nmol/animal), and slightly inhibited by the non-selective serotonin receptor antagonist methysergide (100 nmol/animal). The results suggest that the analgesic action of Neurotropin is at least in part due to the enhancement of noradrenergic and serotonergic descending pain inhibitory pathways. Neurotropin may be useful for the clinical management of chronic pain diseases such as a rheumatoid arthritis and osteoarthritis.

**Keywords:** Neurotropin, adjuvant-induced arthritis, descending pain inhibitory pathway, serotonin receptor,  $\alpha_2$ -adrenoceptor

### Introduction

Neurotropin<sup>®</sup>, a non-protein extract from the inflamed skin of rabbits inoculated with vaccinia virus, has been clinically used as analgesic drugs of injection and tablet forms for treatment of chronic pain such as lumbago, neck-shoulder-arm syndrome, symptomatic neuralgia, postherpetic neuralgia, subacute myelo-optico-neuropathy (1). The usefulness of Neurotropin for complex regional pain syndrome has also been reported (2, 3).

The analgesic effect of Neurotropin has been shown in some animal models, such as SART (specific alternation of rhythm in environmental temperature) stress model (4–6) and CCI (chronic constriction injury) model with painful peripheral neuropathy (7, 8). However, since the analgesic effect of Neurotropin was

discovered in SART-stressed animals first, we mainly investigated the analgesic mechanism using SART-stressed animals. It has been reported that Neurotropin produces a more marked analgesic effect in animals with hyperalgesia induced by SART stress than in normal animals (4–6). Animals given SART stress show the malfunction of the descending pain inhibitory pathway, and the analgesic effect of Neurotropin in this model has been suggested to be due to the enhancement of this pathway (9–13). The monoaminergic descending pathways have been known to control the transmission of nociceptive information in the dorsal horn (14). A combination of Neurotropin and the tricyclic antidepressant amitriptyline produces a synergistic analgesic effect in SART-stressed animals (15). The synergism was speculated to be due to the enhancement of the activation of the monoaminergic descending pathway by Neurotropin with the inhibition of monoamine re-uptake by amitriptyline. Hyperalgesia induced by SART stress

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may be neither a spontaneous pain model nor an inflammatory chronic pain model. On the other hand, hyperalgesia induced by adjuvant-induced arthritis is an inflammatory chronic pain model (16) and may have spontaneous pain. This model has structural damage of nerve fibers (17); therefore, these animals may have neuropathic pain in addition to inflammatory pain. It was unknown whether Neurotropin would inhibit inflammatory chronic pain and whether the descending monoaminergic pathways would be involved in the effect of Neurotropin on this pain. The present study was conducted to address these questions. We have presented this study at the 72nd Annual Meeting of The Japanese Pharmacological Society (18).

## Material and Methods

### Animals

Male Wistar rats (Japan SLC, Hamamatsu) and Sprague-Dawley rats (Japan Charles River, Yokohama) were used; they were nine-week-old at the start of the experiments. The animals were housed at  $22 \pm 2^\circ\text{C}$  under a 12-h light and dark cycle (light turned on from 8:00 to 20:00). Laboratory chow and tap water were available ad libitum. The experiment was done in accordance with Guiding Principles for the Care and Use of Laboratory Animals and the Guidelines for Animal Experimentation approved by The Japanese Pharmacological Society and The Japanese Association for Laboratory Animal Science.

### Drugs

Neurotropin was prepared by Nippon Zoki Pharmaceutical Co. (Osaka). The biological activity of Neurotropin is standardized by the analgesic test in SART-stressed animals and is expressed as Neurotropin Unit (NU). Yohimbine hydrochloride, *m*-chlorophenylbiguanide (*m*-CPBG), methysergide maleate, and MDL72222 were purchased from RBI (Natick, MA, USA). Clonidine hydrochloride was purchased from Sigma (St. Louis, MO, USA).

### Drug administration

Neurotropin was administered intravenously and orally. Neurotransmitter receptor agonist and antagonist were administered intrathecally referring to the method of Hylden and Wilcox using mice (19). Briefly, 10  $\mu\text{l}$  of each solution was injected through a lumbar puncture between L4 and L5 vertebrae using a 30-gauge tuberculin needle. We chose the dosage of the antagonists that had no significant effects on the nociceptive threshold and behaviors in rats on the basis of the previous report (13).

### Induction of adjuvant-induced arthritis

Arthritis was induced by a subcutaneous injection of 0.1 mL of adjuvant (0.5% killed *Mycobacterium butyricum* suspended in liquid paraffin) into the sole of the rat hind paw (20, 21). For drug administration, the rats were used 14 days after adjuvant treatment.

### Measurement of nociceptive threshold

The nociceptive threshold was determined using an analgesy meter (Ugo basile, Milan, Italy). The mechanical pressure stimulus was applied on the center of the hind paw and was increased at a rate of 32 g/s. The pressure intensity (g) that caused the escape reaction was defined as the nociceptive threshold. In the case of the antagonist experiment, the nociceptive threshold was measured before administration of Neurotropin and 15 min after the intrathecal injection of receptor antagonists. The difference in nociceptive threshold (g) between pre-drug and post-drug was expressed as the  $\Delta$  value.

### Statistical analyses

Data were expressed as means  $\pm$  S.E.M. The statistical significance between two groups was determined by Student's *t*-test. Statistical significance between more than two groups was analyzed using Dunnett's or Turkey-Kramer's multiple comparison test. *P* values less than 0.05 were considered to be significant.

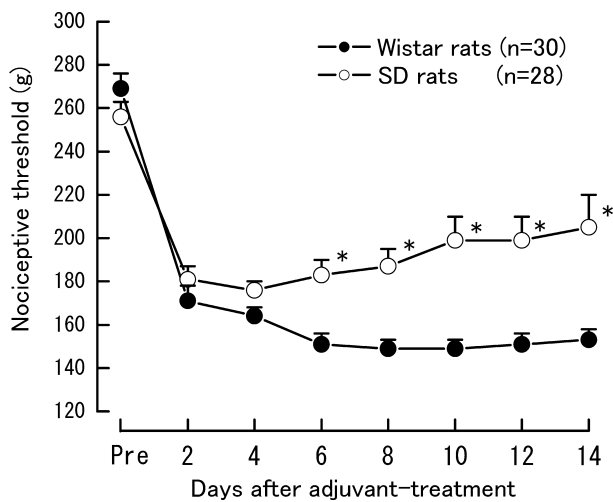
## Results

### Comparison of nociceptive threshold in Wistar and Sprague-Dawley rats after adjuvant treatment

Strain differences of rats have been reported in the arthritis development after adjuvant inoculation (22, 23). Thus, at first, we assessed the strain differences in the severity of hyperalgesia induced by adjuvant inoculation. Adjuvant treatment decreased the nociceptive threshold to a similar extent in Wistar and Sprague-Dawley rats after 2 and 4 days. The nociceptive threshold was relatively constant between 6 and 14 days after adjuvant treatment in Wistar rats, while it gradually elevated after day 6 in Sprague-Dawley rats (Fig. 1). Therefore, we used Wistar rats in the following experiments.

### Analgesic effects of Neurotropin

Analgesic effects of Neurotropin were investigated after intravenous and oral administrations. Neurotropin at an intravenous dose of 10 NU/kg was without effect and higher doses of 30 and 100 NU/kg increased the nociceptive thresholds in a dose-dependent manner; the effects peaked at 30 min and almost subsided at 60 min



**Fig. 1.** Changes in nociceptive threshold after adjuvant treatment in Wistar and Sprague-Dawley (SD) rats. Data are expressed as means  $\pm$  S.E.M. \*Significant difference from Wistar rats at  $P < 0.05$  by Student's *t*-test.

(Fig. 2a). Neurotropin at an oral dose of 30 NU/kg was without effect and higher doses of 100 and 200 NU/kg increased the nociceptive threshold in a dose-dependent manner; the effects peaked at 60 min and subsided by 2 h (Fig. 2b). In normal rats, both Neurotropin at an intravenous dose of 100 NU/kg and an oral dose of 200 NU/kg slightly increased the nociceptive thresholds, but there were no significant differences compared to the normal control group (Fig. 2: a and b).

#### *Influence of yohimbine, an $\alpha_2$ -adrenoceptor antagonist, on the analgesic effect of Neurotropin*

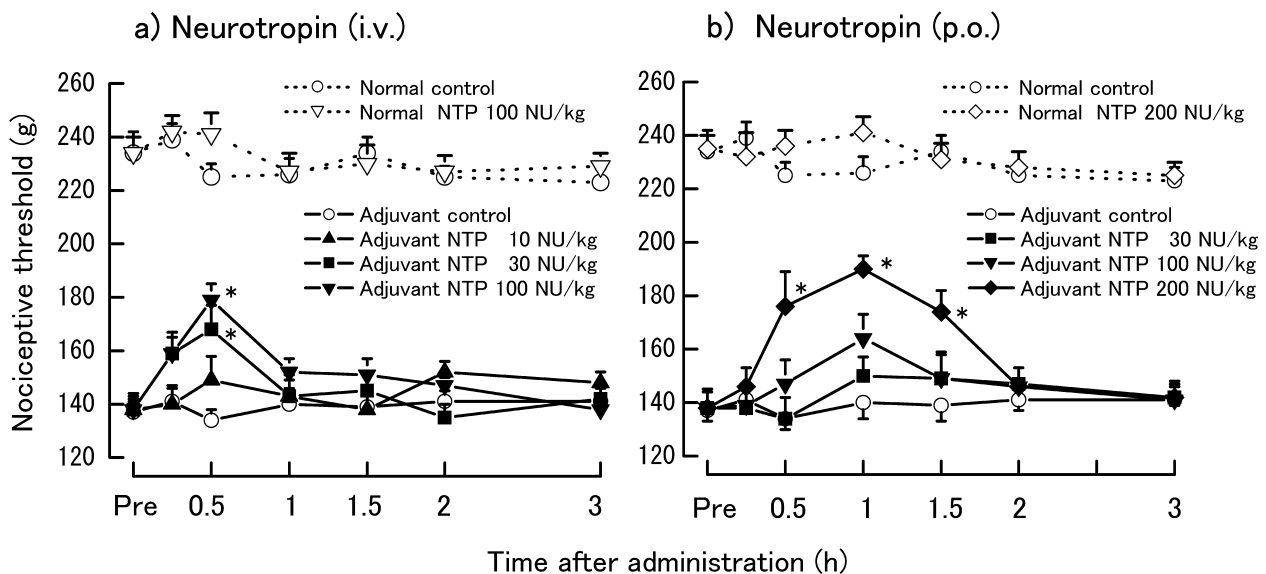
Neurotropin at an intravenous dose of 100 NU/kg and an oral dose of 200 NU/kg increased the nociceptive threshold to a similar extent in the arthritic rats. Yohimbine at an intrathecal dose of 30 nmol/animal almost completely abolished the effect of intravenous and oral Neurotropin (Fig. 3). Intrathecal yohimbine (30 nmol/animal) alone did not affect the nociceptive threshold of the arthritic rats (Fig. 3).

#### *Influence of serotonergic antagonists on the analgesic effect of Neurotropin*

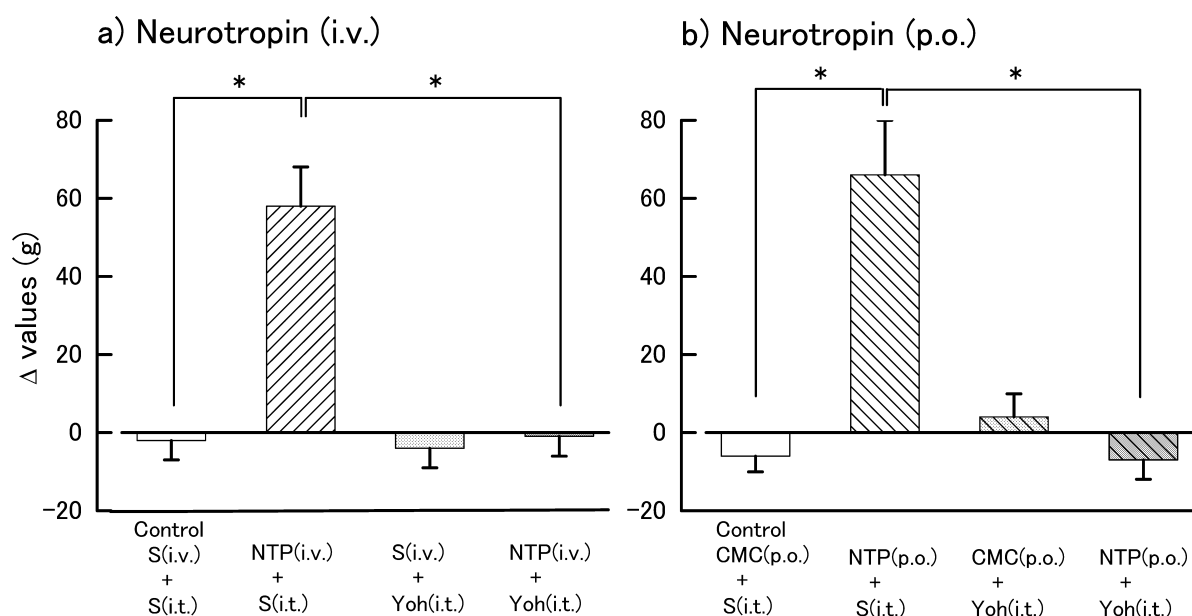
Methysergide at an intrathecal dose of 100 nmol/animal produced slight and non-significant inhibition of the increase of nociceptive threshold induced by intravenous and oral administration of Neurotropin at 100 and 200 NU/kg, respectively (Fig. 4). MDL72222 at an intrathecal dose of 30 nmol/animal produced the substantial inhibition of the increase of nociceptive threshold induced by intravenous and oral Neurotropin at 100 and 200 NU/kg, respectively (Fig. 5).

#### *Analgesic effects of noradrenergic and serotonergic receptor agonists*

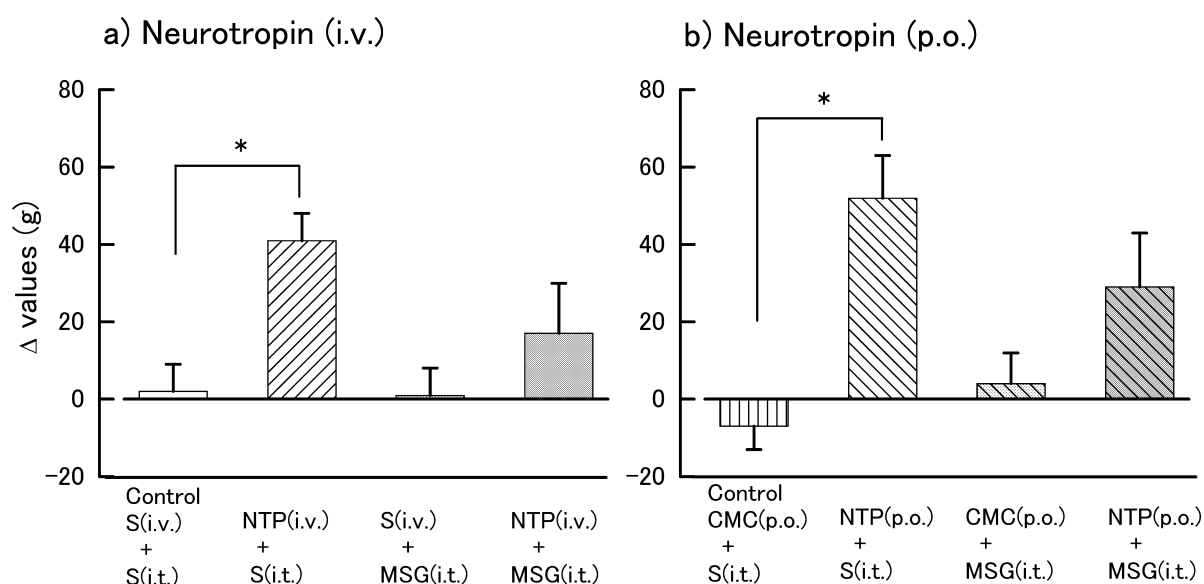
The  $\alpha_2$ -adrenoceptor agonist clonidine at an intrathecal dose of 100 nmol/animal significantly increased the nociceptive threshold, and lower doses of 10 and 30 nmol/animal were without effects in normal rats (Fig. 6a). In arthritic rats, clonidine at intrathecal doses



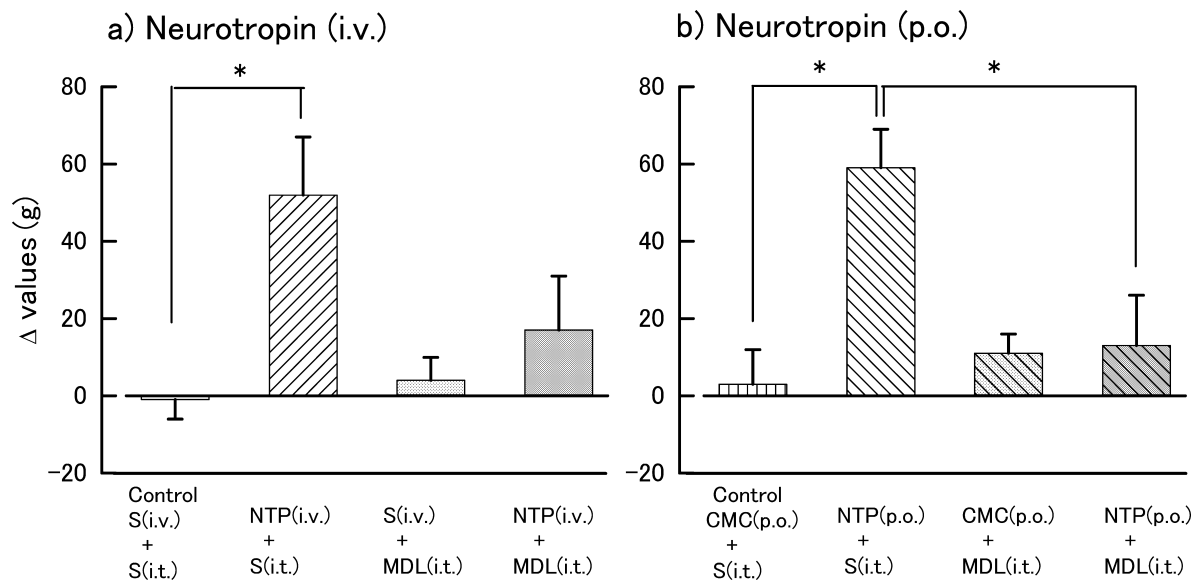
**Fig. 2.** The analgesic effects of intravenous (a) and oral (b) administration of Neurotropin in normal and adjuvant-induced arthritic rats. Male Wistar rats were used. Data are expressed as means  $\pm$  S.E.M. ( $n = 8$ ). \*Significant difference from the adjuvant control group at  $P < 0.05$  by Dunnett's multiple comparison test. NTP: Neurotropin.



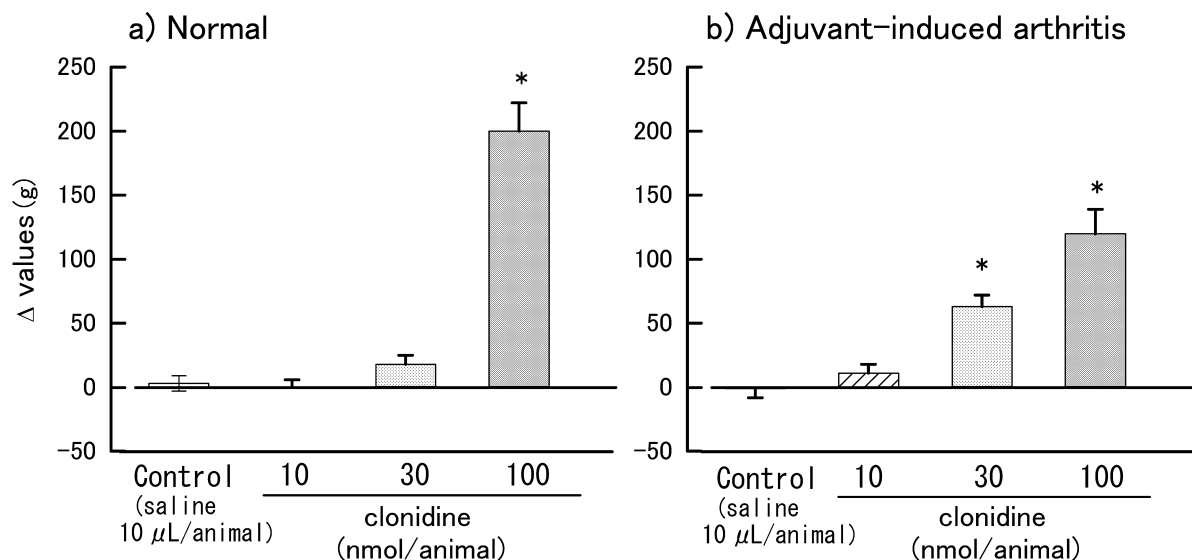
**Fig. 3.** Influence of intrathecal injection of yohimbine on the analgesic effect of Neurotropin in rats with adjuvant-induced arthritis. Male Wistar rats were used. The nociceptive threshold was measured before administration, 30 min after intravenous and 60 min after oral Neurotropin. Yohimbine was intrathecally injected 15 min before measuring. The differences ( $\Delta$  values) of nociceptive threshold between before and after administration of drugs were calculated. Data are expressed as means  $\pm$  S.E.M. ( $n = 7$ ). \*Significant difference from each control group at  $P < 0.05$  by Tukey-Kramer multiple comparison test. S(i.v.), saline at 10 mL/kg (intravenous); NTP(i.v.), Neurotropin at 200 NU/kg (intravenous); CMC(p.o.), 0.5% CMC at 10 mL/kg (per os); NTP(p.o.), Neurotropin at 200 NU/kg (per os); S(i.t.), saline at 10  $\mu$ L/rat (intrathecal); Yoh(i.t.), yohimbine at 30 nmol/rat (intrathecal).



**Fig. 4.** Influence of intrathecal injection of methysergide on the analgesic effect of Neurotropin in rats with adjuvant-induced arthritis. Male Wistar rats were used. The nociceptive threshold was measured before administration, 30 min after intravenous and 60 min after oral Neurotropin. Methysergide was intrathecally injected 15 min before measuring. The differences ( $\Delta$  values) of nociceptive threshold between before and after administration of drugs were calculated. Data are expressed as means  $\pm$  S.E.M. ( $n = 7$ ). \*Significant difference from each control group at  $P < 0.05$  by Tukey-Kramer multiple comparison test. S(i.v.), saline at 10 mL/kg (intravenous); NTP(i.v.), Neurotropin at 100 NU/kg (intravenous); CMC(p.o.), 0.5% CMC at 10 mL/kg (per os); NTP(p.o.), Neurotropin at 200 NU/kg (per os); S(i.t.), saline at 10  $\mu$ L/rat (intrathecal); MSG(i.t.), methysergide at 100 nmol/rat (intrathecal).



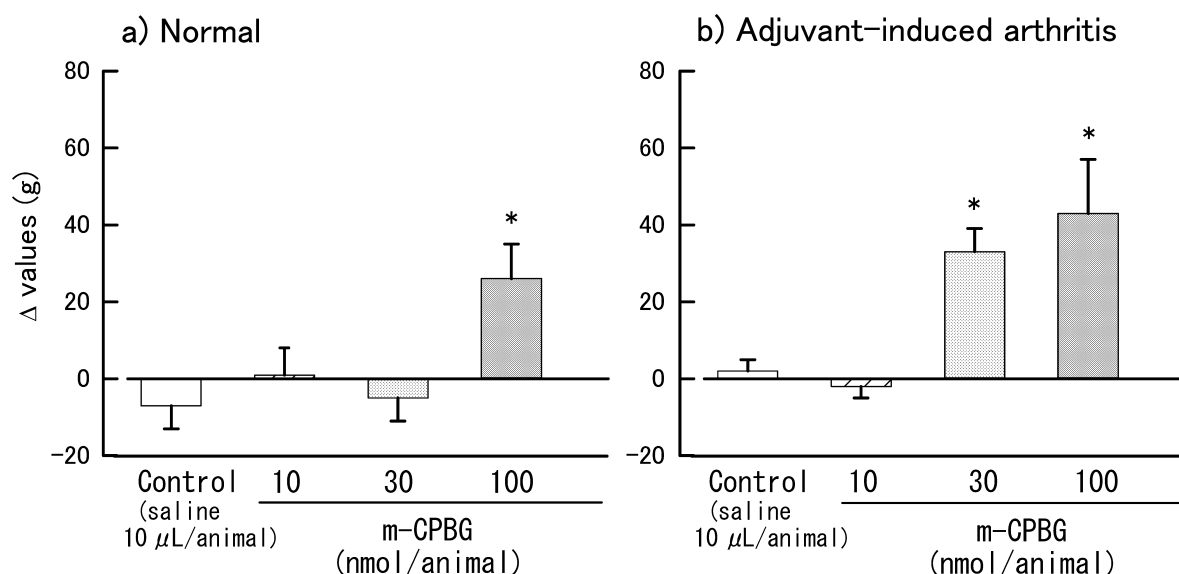
**Fig. 5.** Influence of intrathecal injection of MDL72222 on the analgesic effect of Neurotropin in rats with adjuvant-induced arthritis. Male Wistar rats were used. The nociceptive threshold was measured before administration, 30 min after intravenous and 60 min after oral Neurotropin. MDL72222 was intrathecally injected 15 min before measuring. The differences ( $\Delta$  values) of nociceptive threshold between before and after administration of drugs were calculated. Data are expressed as means  $\pm$  S.E.M. ( $n = 7$ ). \*Significant difference from each control group at  $P < 0.05$  by Tukey-Kramer multiple comparison test. S(i.v.), saline at 10 mL/kg (intravenous); NTP(i.v.), Neurotropin at 100 NU/kg (intravenous); CMC(p.o.), 0.5% CMC at 10 mL/kg (per os); NTP(p.o.), Neurotropin at 200 NU/kg (per os); S(i.t.), saline at 10  $\mu$ L/rat (intrathecal); MDL(i.t.), MDL72222 at 30 nmol/rat (intrathecal).



**Fig. 6.** Influence of intrathecal injection of clonidine on nociceptive thresholds in normal and adjuvant-induced arthritic rats. Male Wistar rats were used. The nociceptive threshold was measured 15 min after intrathecal clonidine. The differences ( $\Delta$  values) of nociceptive threshold between before and after injection of clonidine were calculated. Data are expressed as means  $\pm$  S.E.M. ( $n = 7$ ). \*Significant difference from each control group at  $P < 0.05$  by Dunnett's multiple comparison test.

of 30 and 100 nmol/animal dose-dependently increased the nociceptive threshold (Fig. 6b). The increase of nociceptive threshold after clonidine at a dose of

30 nmol/animal was larger in arthritic rats than in normal ones and the increase after a dose of 100 nmol/animal was larger in normal rats.



**Fig. 7.** Influence of intrathecal injection of m-CPBG on nociceptive thresholds in normal and adjuvant-induced arthritic rats. Male Wistar rats were used. The nociceptive threshold was measured 15 min after intrathecal m-CPBG. The differences ( $\Delta$  values) of nociceptive threshold between before and after injection of m-CPBG were calculated. Data are expressed as means  $\pm$  S.E.M. ( $n = 7$ ). \*Significant difference from each control group at  $P < 0.05$  by Dunnett's multiple comparison test.

The selective 5-HT<sub>3</sub> serotonin receptor agonist m-CPBG at an intrathecal dose of 100 nmol/animal produced a significant increase in nociceptive threshold, and lower doses of 10 and 30 nmol/animal were without effects in normal rats (Fig. 7a). In arthritic rats, m-CPBG at intrathecal doses of 30 and 100 nmol/animal dose-dependently increased the nociceptive threshold and a lower dose of 10 nmol/animal was without effect (Fig. 7b).

## Discussion

The noradrenergic descending inhibitory pathway is known to play an important role in endogenous analgesic systems, which project from A5 and A7 cell groups of the pons to the spinal dorsal horn (24). Therefore, we examined the involvement of the descending noradrenergic system in the regulation of chronic arthritic pain and analgesic effect of Neurotrophin. Clonidine, an  $\alpha_2$ -adrenoceptor agonist, was injected intrathecally into normal rats and adjuvant-induced arthritic rats. Intrathecal clonidine showed a clear analgesic effect in both rats with algnesia induced by mechanical stimulation. Other groups reported that intrathecal clonidine alleviated thermal hyperalgesia in adjuvant-induced arthritic rats (25) and that increase in spinal excitatory amino acid was suppressed by an  $\alpha_2$ -adrenoceptor agonist in this model (26). These findings and our results suggest that the spinal  $\alpha_2$ -adrenoceptor plays an important role in alleviating both thermal and

mechanical hyperalgesia.

The analgesic effects of intravenous and oral Neurotrophin were significantly inhibited by an intrathecal injection of yohimbine, an  $\alpha_2$ -adrenoceptor antagonist. Taken together with the results of clonidine experiments, the results suggest the important role of the activation of noradrenergic descending pain inhibitory pathway in the inhibition of arthritic hyperalgesia by Neurotrophin.

The involvement of the descending serotonergic system in the analgesic effect of Neurotrophin on the arthritic hyperalgesia was also examined. The suppression of arthritic hyperalgesia by oral Neurotrophin was significantly inhibited by an intrathecal injection of the selective 5-HT<sub>3</sub> serotonin receptor antagonist MDL72222. The effect of intravenous Neurotrophin was also markedly, but not significantly, inhibited by MDL72222. These results suggest the involvement of 5-HT<sub>3</sub> serotonin receptor and descending serotonergic system in the Neurotrophin action. An intrathecal injection of the selective 5-HT<sub>3</sub> receptor agonist m-CPBG significantly elevated nociceptive threshold, which was more marked in rats with adjuvant-induced arthritis than in normal rats. The results are consistent with the report that 5-HT<sub>3</sub> serotonin receptors are involved in the modulation of spinal nociceptive reflex (27) and suggest that 5-HT<sub>3</sub> serotonin receptors play a more important role in the hyperalgesic state than in healthy state.

The analgesic effect of Neurotrophin in rats with adjuvant-induced arthritis was slightly, but not significantly, inhibited by methysergide. This agent is a non-

selective 5-HT receptor antagonist and acts even as an agonist (28, 29). For example, methysergide acts as an antagonist on 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> serotonin receptors, but as an agonist on 5-HT<sub>1A/1B</sub> and 5-HT<sub>1D</sub> serotonin receptors (30, 31). With these findings taken into account, the present results suggest that 5-HT<sub>1,2</sub> serotonin receptors in the spinal dorsal horn do not play an important role in the analgesic action of Neurotropin. Some neurochemical studies have suggested that adjuvant arthritis affects the descending monoaminergic systems. In detail, the spinal content of serotonin is increased about 2 weeks after adjuvant treatment (32, 33). However, in the other report, there was no change in the spinal cord (34). These reports indicate that a change in spinal content of serotonin is unclear at 2 weeks after adjuvant treatment, which is the time period that we used. Accompanying with our results, the expression or the activity of serotonin receptor subtypes might be changed.

On the other hand, SART-stressed animals show chronic hyperalgesia (35, 36). SART stress does not produce inflammation, and decreased function of descending pain inhibitory systems may play a role in the SART stress hyperalgesia (9–13). Thus, the pathogenesis of hyperalgesia may be different between SART stress and adjuvant arthritis. However, adjuvant-induced arthritic rats with chronic pain may also have the dysfunction of descending pain inhibitory systems. Thus, Neurotropin may alleviate hyperalgesia through the activation of descending pain inhibitory systems regardless of different pathogenesis of hyperalgesia. Amitriptyline and imipramine, monoamine re-uptake inhibitors, have also been shown to clearly reduce pain behavior in adjuvant-induced arthritic rats (37, 38). These reports support that the activation of the descending pain inhibitory pathway is useful for relief of chronic pain.

In conclusion, Neurotropin inhibited chronic hyperalgesia induced by adjuvant arthritis, suggesting that Neurotropin is useful for the clinical management of chronic pain diseases such as a rheumatoid arthritis or osteoarthritis. The inhibition of hyperalgesia by Neurotropin may be at least in part due to the enhancement of descending pain inhibitory, especially noradrenergic and serotonergic, pathways.

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