Mechanism of Hyperalgesia in SART Stressed (Repeated Cold Stress) Mice: Antinociceptive Effect of Neurotropin

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ABSTRACT — Exposing mice to 24 and 4°C in alternate 1 hr periods in the day time and maintaining 4°C at night for several days decreases the tail clamp pressure required to evoke pain behavior. This model is referred to as SART (specific alternation rhythm of temperature) stress. An extract from inflamed skin of rabbits inoculated with vaccinia virus (neurotropin) clearly normalized the hyperalgesia in this SART stress model. To clarify the mechanism of the hyperalgesia in SART mice and the mode of the antinociceptive action of neurotropin in this model, the influence of systemically administered neurotransmitter related drugs was studied. 1) Neurotropin, 5-hydroxytryptophan and L-dihydroxyphenylalanine significantly normalized the decrease in nociceptive threshold, and muscimol tended to inhibit it in nociceptive threshold in SART stressed mice. 2) Haloperidol, phenoxybenzamine, reserpine, bicuculline, scopolamine, physostigmine and naloxone alone did not influence the nociceptive threshold in SART stressed mice. 3) The antinociceptive effect of neurotropin was significantly attenuated by p-chlorophenylalanine, haloperidol and phenoxybenzamine; and it was completely inhibited by reserpine. 4) Naloxone, bicuculline, scopolamine and physostigmine had no influence on the antinociceptive effect of neurotropin. These results suggest that hypofunction mainly of the monoaminergic systems contributes to hyperalgesia in SART stressed mice and that neurotropin produces the antinociceptive effect by restoring these neural functions.

There are many reports on the influence of stress on the physiological functions of animals. SART (specific alternation rhythm of temperature) stressed mice become hyperalgesic (1–5), in contrast to the so-called “stress induced analgesia” caused by acute stress, such as electrical shock, cold water swim, restraint, etc. (6). In addition, the hyperalgesia of SART stressed mice lasts for as long as one week after removal of the stress (1). SART stressed animals might thus be considered to be a chronic pain model.

Neurotropin, a non-protein extract from the inflamed skin of rabbits inoculated with vaccinia virus, has been used in Japan for the therapy of chronic pain such as post herpetic neuralgia and low back pain. In experimental animals, it has been reported that neurotropin produces remarkably more analgesia in SART stressed animals than in normal animals (2, 4, 5). Little is known about the mechanism of the antinociceptive effect of neurotropin. It is
now known that endogenous pain inhibitory systems, such as the monoaminergic, opioidergic and GABAergic systems, modulate the pain sensation in the central nervous system (7–9). To verify the mechanisms of the antinociceptive effect of neurotropin, we examined the influence of neurotransmitter related drugs on hyperalgesia in SART stressed mice and on the antinociceptive effect of neurotropin.

MATERIALS AND METHODS

Animals and stress loading
Male 4-week old ddY mice (purchased from SLC, Shizuoka, Japan) were used for the experiments (7–8 animals per group). Prior to the experiments, they were kept under constant temperature (24 ± 1°C), humidity (55 ± 10%) and artificial 08:00 to 20:00 lighting conditions with ad libitum access to food and water.

As described by Hata et al. and Kita et al. (10, 11), the animals were kept at 24°C and 4°C for alternate 1 hr periods from 10:00 to 17:00 and then at 4°C from 17:00 to 10:00 the following morning. This procedure was repeated for 7 consecutive days.

Drugs and administration
L-5-Hydroxytryptophan (5-HTP) was purchased from Nacalai Tesque (Japan). L-Dihydroxyphenylalanine (L-DOPA), haloperidol, DL-p-chlorophenylalanine (PCPA), reserpine and scopolamine hydrobromide were purchased from Wako Pure Chemical Industries (Japan). Naloxone was purchased from U.S.P.C. (U.S.A.). Physostigmine sulfate and phenoxybenzamine hydrochloride were purchased from Tokyo Kasei (Japan). Muscimol and bicuculline were purchased from Sigma (U.S.A.). Neurotropin, a non-proteinaceous extract containing biologically active small molecules (molecular weights of less than 2,000) isolated from the inflamed skin of rabbits inoculated with vaccinia virus (12), was prepared by Nippon Zoki.

Drugs were suspended in 0.5% sodium carboxymethylcellulose/saline, except for bicuculline which was dissolved in acidic saline (pH 3.0) adjusted with hydrochloric acid as described by Olsen et al. (13).

Test drugs were administered between 14:00 and 15:00 at 24°C once daily during the stress period, except for PCPA (every 3 days: days 0, 3 and 6). Drugs were administered s.c., except for neurotropin (i.p.) and 5-HTP (p.o.). The control group was treated with vehicle intraperitoneally. When studying the influences of neurotransmitter related drugs on the antinociceptive effect of neurotropin, they were administered immediately after neurotropin.

Measurement of mechano-nociceptive threshold
Mechano-nociceptive threshold was measured by the tail pressure method (4) between 10:00 and 11:00 in 24°C ambient temperature. A pressure point of the Randall-Selitto apparatus (Ugo Basile, Italy) was changed to a plastic plate (1.5 mm thick and 2.0 cm wide), and mechanical pressure was applied about 1.5 cm caudal from the base of the tail at a rate of 16 g/sec. The pressure intensity that caused an escape reaction was defined as the nociceptive threshold (g). Prior to the administration of drugs, the nociceptive threshold was measured twice with a 30 min interval. Mice showing a mean nociceptive threshold below 250 g were used in the experiments. In the administration period, the nociceptive threshold was measured once a day, every day.

Effects of drugs on hyperalgesia in SART mice and influences of drugs on the antinociceptive effect of neurotropin were evaluated on days 5–7, because SART mice remained stably hyperalgesic in these periods (see Figs. 1 and 2).

Statistical analysis
The data, expressed as the mean ± S.E., were analyzed by Student's t-test or two-way analysis of variance (ANOVA). Differences with P values less than 0.05 were considered to be statistically significant.
RESULTS

Influence of neurotransmitter related drugs on hyperalgesia in SART mice

The nociceptive threshold of normal control mice ranged from 120 ± 8 to 130 ± 8 g (99 ± 6 to 106 ± 5% compared to before stress) during the experimental period. The nociceptive threshold of SART stressed mice decreased significantly starting one day after onset of the stress and was at most 92 ± 6 g (77 ± 6%) on and after the 4th day. Neurotropin normalized the decreased nociceptive threshold of SART stressed mice dose-dependently at 20 and 100 mg/kg/day, and 100 mg/kg of neurotropin completely reversed the effect of SART (Fig. 1a).

The nociceptive threshold of stressed mice decreased significantly.

![Fig. 1. Effects of daily systemic administration of neurotropin, 5-HTP, L-DOPA and muscimol on the mechano-nociceptive threshold in SART stressed mice. Each point and vertical bar shows the mean ± S.E. of the change in nociceptive threshold compared to that before stress (n = 8). *P < 0.05 vs. SART stressed control (days 5-7, ANOVA).](image-url)
was dose-dependently increased by 10 and 50 mg/kg/day of 5-HTP, a precursor of serotonin (5-HT); and 50 mg/kg of 5-HTP completely reversed the effect of SART (Fig. 1b). L-DOPA (20 and 100 mg/kg/day), a precursor of catecholamine, significantly increased the nociceptive threshold from the 5th day after the onset of stress (Fig. 1c). Muscimol (0.1 and 0.5 mg/kg/day), a GABAA agonist, slightly increased the nociceptive threshold of stressed mice (Fig. 1d).

Haloperidol, an antagonist of dopamine, and phenoxybenzamine, a noradrenergic α-antagonist, at the dose of 0.2 or 1.0 mg/kg/day, had slight or no influence on the nociceptive threshold of stressed mice. Naloxone (0.5 and 2 mg/kg/day), an opioid antagonist; physostigmine (0.02 and 0.1 mg/kg/day), a cholinesterase inhibitor; and scopolamine (0.5 and 2 mg/kg/day), a muscarinic antagonist, had no influence on the nociceptive threshold of stressed mice (Table 1).

### Table 1. Normalizing effects of neurotransmitter related drugs on the decreased mechano-nociceptive threshold in SART stressed mice

| Drug                  | Dose (mg/kg) | Route | Changes in NT (%) | Normalization |
|-----------------------|-------------|-------|-------------------|---------------|
|                       |             |       | 5 days            | 6 days        | 7 day         |               |
| Normal control        |             |       | 104 ± 4           | 100 ± 3       | 105 ± 4       |               |
| SART control          |             |       |                   |               |               |               |
| Neurotropin           | 20          | i.p.  | 73 ± 3            | 71 ± 3        | 72 ± 4        |               |
|                       | 100         | i.p.  | 94 ± 3            | 88 ± 6        | 90 ± 3        |               |
| 5-HTP                 | 10          | p.o.  | 104 ± 7           | 103 ± 6       | 103 ± 7       |               |
|                       | 50          | p.o.  | 91 ± 7            | 92 ± 6        | 88 ± 6        |               |
| L-DOPA                | 20          | s.c.  | 104 ± 4           | 103 ± 6       | 102 ± 6       |               |
|                       | 100         | s.c.  | 88 ± 5            | 95 ± 4        | 93 ± 4        |               |
| Haloperidol           | 0.2         | s.c.  | 71 ± 3            | 71 ± 6        | 74 ± 4        |               |
|                       | 1           | s.c.  | 77 ± 6            | 76 ± 4        | 81 ± 7        |               |
| Phenoxybenzamine      | 0.2         | s.c.  | 72 ± 5            | 80 ± 4        | 81 ± 4        |               |
|                       | 1           | s.c.  | 84 ± 7            | 83 ± 3        | 75 ± 4        |               |
| Reserpine             | 0.1         | s.c.  | 71 ± 3            | 62 ± 3        | 69 ± 3        |               |
|                       | 0.3         | s.c.  | 68 ± 5            | 62 ± 4        | 64 ± 5        |               |
| Naloxone              | 0.5         | s.c.  | 69 ± 2            | 69 ± 2        | 68 ± 3        |               |
|                       | 2           | s.c.  | 76 ± 7            | 68 ± 6        | 69 ± 4        |               |
| Muscimol              | 0.1         | s.c.  | 83 ± 5            | 85 ± 6        | 80 ± 5        |               |
|                       | 0.5         | s.c.  | 82 ± 6            | 77 ± 4        | 72 ± 4        |               |
| Bicuculline           | 0.1         | s.c.  | 75 ± 5            | 71 ± 5        | 71 ± 4        |               |
|                       | 0.5         | s.c.  | 74 ± 6            | 80 ± 5        | 74 ± 6        |               |
| Physostigmine         | 0.02        | s.c.  | 80 ± 3            | 74 ± 3        | 72 ± 6        |               |
|                       | 0.1         | s.c.  | 80 ± 6            | 76 ± 5        | 75 ± 6        |               |
| Scopolamine           | 0.5         | s.c.  | 71 ± 5            | 82 ± 7        | 81 ± 5        |               |
|                       | 2           | s.c.  | 73 ± 4            | 73 ± 5        | 65 ± 5        |               |

Data show the mean ± S.E. of the change in nociceptive threshold (NT) compared to that before stress (n = 8). *P < 0.05 vs. each SART stressed control (t-test). † or →: P < 0.05 or not significant vs. SART stressed control, respectively (days 5−7, ANOVA).
significantly attenuated the antinociceptive effect of neurotropin. Reserpine (0.3 mg/kg/day) completely attenuated the antinociceptive effect of neurotropin (Fig. 2d).

Neither bicuculline (0.5 mg/kg/day), a GABA_A antagonist; naloxone (2 mg/kg/day); scopolamine (2 mg/kg/day); nor physostigmine (0.1 mg/kg/day) modified the effect of neurotropin (Table 2).

DISCUSSION

It has been reported that neurotropin produces little or no antinociceptive effect in normal mice, but clearly normalizes SART stressed hyperalgesic mice (2, 4, 5). The aim of the present experiment was the clarification of the mechanisms of the hyperalgesia caused by SART stress and the antinociceptive effect of
neurotropin. In the first of the present experiments, neurotransmitter related drugs were administered daily to investigate the neural mechanism of hyperalgesia in SART stressed mice.

Table 2. Effects of neurotransmitter related drugs on antinociceptive activity of neurotropin in SART stressed mice

| Drug                                | Dose (mg/kg) | Route | Changes in NT (%) | Attenuation |
|-------------------------------------|--------------|-------|-------------------|-------------|
| Neurotropin                         | 100          | i.p.  | 89 ± 9            |             |
| Combined administration with neurotropin |              |       | 89 ± 6 | 98 ± 7 |
| PCPA                                | 300          | s.c.  | 81 ± 7            | ↓           |
| Haloperidol                         | 1            | s.c.  | 73 ± 3            | ↓           |
| Phenoxybenzamine                    | 1            | s.c.  | 77 ± 5            | ↓           |
| Reserpine                           | 0.3          | s.c.  | 59 ± 5            | ↓           |
| Naloxone                            | 2            | s.c.  | 87 ± 4            | →           |
| Bicuculline                         | 0.5          | s.c.  | 86 ± 8            | →           |
| Scopolamine                         | 2            | s.c.  | 97 ± 4            | →           |
| Physostigmine                       | 0.1          | s.c.  | 83 ± 5            | →           |

Data show the mean ± S.E. of the change in nociceptive threshold (NT) compared to that before stress (n = 7). ↓ or →: P < 0.05 or not significant vs. neurotropin group, respectively (days 5–7, ANOVA).

The hyperalgesia in SART mice was suppressed almost completely by systemic administration of 5-HP, a precursor of 5-HT, and partially suppressed by L-DOPA, a precursor of catecholamine, from the 5th day after the start of stress. Similar dosed of these drugs did not affect the nociceptive threshold in normal mice (data are not shown). It is well known that monoamines are related to pain regulation in the central nervous system (7), and especially 5-HT and noradrenaline (NA) are involved in the endogenous central antinociceptive system (8, 9, 14). The descending serotonergic system, projecting from the nucleus raphe magnus to the spinal dorsal horn mediates pain inhibition (7–9, 15). Post et al. (16) reported analgesia in rats treated with 5-HP. It has also been reported that the antinociceptive effects of morphine or pentazocine were attenuated by pretreatment with PCPA, an inhibitor of 5-HT biosynthesis, or 5,6-dihydroxytryptamine (17). NA is also related to the descending pain inhibitory system (7, 9, 14), although cerebroventricular injection of NA antagonizes opioid analgesia (18). The hyperalgesia in SART mice was slightly suppressed by the daily treatment with a low dose of muscimol (0.1 mg/kg/day) from the 5th day after the start of stress. GABA is thought to inhibit pain transmission in the spinal cord (7). In addition, muscimol potentiates morphine induced analgesia (19), and aminooxysitc acid, an inhibitor of GABA transaminase, prolongs morphine induced analgesia (20). There is also a report that subconvulsive doses of bicusculine strongly attenuate morphine analgesia (20). These findings support the idea that serotonergic, noradrenergic and GABAergic systems are also concerned with the pain regulatory system. Therefore, our data suggest that hypofunction of the pain inhibitory monoaminergic and GABAergic systems may be relevant to the hyperalgesia of SART stressed mice.

In the second of the present experiments, the influence of neurotransmitter related drugs on the antinociceptive effect of neurotropin was investigated. The antinociceptive effect of neurotropin in SART mice was attenuated completely by reserpine, an intraneural storage inhibitor of amines, and partially attenu-
ated by PCPA, haloperidol and phenoxybenzamine on and after the 5th day of repeated administration. These results suggest that neurotropin produces its antinociceptive effect by reversing the hypofunction of the pain inhibitory system, at least the monoaminergic systems. Since the antinociceptive effect of neurotropin by an intracisternal route was 100 times that of an i.p. injection, neurotropin may act at central levels (21). The antinociceptive effect of neurotropin was not affected by naloxone in this experiment. Hata et al. also reported that the antinociceptive effect of neurotropin was not blocked by a single administration of naloxone, and no cross-tolerance developed between neurotropin and morphine (21). The function of neurotropin is different from that of aspirin in formalin induced hyperalgesic mice (22) and from that of indomethacin in the inhibition of inflammatory mediator release by paw perfusion (23). From this evidence, the mode of action of neurotropin seems to differ from that of narcotics or non-steroidal antiinflammatory drugs. Further study is needed to clarify which neurons are altered by SART stress, and restored by neurotropin.

In conclusion, one of the mechanisms of hyperalgesia in SART stressed mice is thought to be dysfunction of the pain inhibitory systems including the monoaminergic systems; and hyperalgesia is restored by treatment with neurotropin.

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