Mechanism of the Analgesic Effect of Neurotropin

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Accepted June 13, 1988

Abstract—Neurotropin, an extract from the inflamed skin of vaccinia virus-inoculated rabbits, has been observed clinically to be effective for treating pain in patients with lumbago, SMON and other neuropathies. In the present study, we examined the mechanism of the antinociceptive effect of neurotropin in mice in relation to administration routes, opioids, and noradrenergic or GABAergic drugs, by the tail pressure method. The antinociceptive effects of neurotropin were large when administered by the i.p. and intracisternal (i.cist.) routes, but comparatively small in the case of the intrathecal (i.th.) route. Neurotropin may thus act at the supraspinal level rather than on the spinal cord. The antinociceptive effect of neurotropin was not blocked by naloxone, and no cross-tolerance developed between neurotropin and morphine. The effect of neurotropin was blocked by phentolamine and reserpine, but not by atropine. Its effect was enhanced by GABA, muscimol, aminoxyacetic acid and diaminobutyric acid, but not by baclofen, and blocked by bicuculline methiodide. From these results, the antinociceptive action of neurotropin appears to be non-opioid in nature, and may possibly be mediated by the noradrenergic and GABAergic systems, but unrelated to the cholinergic system.

Neurotropin, an extract from the inflamed skin of vaccinia virus-inoculated rabbits, has been clinically reported to show analgesic effects in patients with severe pain (1-5) such as that occurring in cases of lumbago, cervicodynia, SMON and various neuropathies. Thus, neurotropin can be effectively used to treat patients with chronic pain, but not those with acute pain. In animal experiments, neurotropin has been reported to exert strong antinociceptive effects (6) on hyperalgesia (7, 8) in SART (specific alternation of rhythm in temperature)-stressed (repeated cold-stressed) mice (9, 10), which show symptoms of vagotonic-type dysautonomia (11), and in other types of hyperalgesic mice (12).

However, the mechanism of its analgesic effect has so far not been clarified. In the present study, therefore, the antinociceptive effects of neurotropin on mice were investigated in relation to administration route, opioids, and noradrenergic or GABAergic agents. Neurotropin was administered to mice centrally or peripherally.

Materials and Methods

Male ddY mice were used. They were housed at 22–24°C under a regime with a constant day-night rhythm and given food and water ad libitum. Normal healthy mice were used in most of the experiments, while in some experiments, SART-stressed mice were employed.

For SART stress loading, the mice were kept alternately at 24°C and 4°C at 1-hr intervals from 9 a.m. to 4 p.m. and then at 4°C from 4 p.m. to 9 a.m. the following morning. This procedure was repeated for 5 consecutive days (9, 10) and then stopped on the morning of the 6th day of stress. The stressed animals were subjected to experiments 1 hr or more after the cessation of stress. Drugs were administered intracisternally, intrathecally or intraperitoneally.

Intracisternal (i.cist.) injection was carried
out using a J-shaped needle (27 gauge) with a 40° curve 3.5 mm from the tip, according to the method of Ueda et al. (13), using mice weighing about 20 g. Each mouse was held manually for this purpose. The needle was inserted into the cleft between the occiput and atlas vertebra through the intact skin. The drug solution was injected at a volume of 10 μl.

For administration of drugs directly into the spinal subarachnoid space, mice weighing about 25 g were given intrathecal (i.th.) injections (14) by lumbar puncture, directing a needle (30 gauge) into an intravertebral space approximately at the level of the 5th or 6th lumbar vertebra. The injection volume was 5 μl.

The nociceptive threshold of the mice was determined by the modified tail pressure method using a Randall-Selitto Analgesy Meter (Ugo Basile). Mice were subjected to a force on the tail at a point 1 cm distant from the root. The force applied to the tail was increased at a constant rate of 16 g/sec. The force required to produce the escape reaction in a mouse was defined as the nociceptive threshold. The ratio of this force after treatment to that before treatment was defined as the antinociceptive index.

To assess the development of cross-tolerance between morphine and neurotropin, their antinociceptive effects were examined on the 4th day in mice rendered tolerant by 3 daily treatments with 10 mg/kg/day of morphine, i.p., according to Kaneto and Kihara (15).

The following drugs were used: Neurotropin® (Nippon Zoki, 20 mg/ml), morphine hydrochloride (Takeda), naloxone hydrochloride (Sigma), phentolamine mesylate (Ciba-Geigy, Japan, Regitin®), reserpine, atropine sulfate (Wako), γ-aminobutyric acid (GABA, Ono, Gammaron®), bicuculline methiodide (Sigma), aminoxyacetic acid (Wako), diaminobutyric acid (Aldrich), muscimol (Sigma), baclofen (Ciba-Geigy, Japan). All were dissolved or diluted in physiological saline or pure water, except for reserpine, which was suspended in 0.5% Tween 80 solution.

Drug effects were observed twice at 2 and 5 min, 5 and 8 min, and 45 and 60 min after i.cist., i.th. and i.p. administration, respectively, and the means of 2 respective values were used as data. When the combined effects of 2 drugs were observed, the 2 drugs were simultaneously administered or one of them was preadministered at an appropriate time in order to exhibit steady effects. When 2 drugs were simultaneously administered, the mixed solutions of 2 drugs were used.

The regression line was calculated by the method of least squares. All values were expressed as means with S.E. Data were analyzed by one-way or two-way analysis of variance (ANOVA). Differences between means were analyzed by Student’s t-test and by ANOVA followed by the Newman-Keuls test, and the interaction between two drugs was assessed by two-way ANOVA. A value of P<0.05 was considered statistically significant.

Results
1. Comparison of antinociceptive effects of neurotropin administered by i.p., i.cist. and i.th. routes to non-stressed and SART-stressed mice (Fig. 1)

Neurotropin showed dose-dependent antinociceptive effects when administered by any of the routes. The effects in SART-stressed mice were significantly greater than those in non-stressed mice, especially by the i.p. and i.cist. routes. Also, significantly larger effects were produced by the i.cist. route than by the i.th. route at any of the corresponding doses, not only in non-stressed mice but also in SART-stressed mice. Neurotropin doses larger than 4 mg/kg were not examined using the i.th. route, because the administration volume was excessive for mice.

2. Antinociceptive effect of neurotropin, non-opioid?

The antinociceptive effect of neurotropin was compared with that of morphine, with or without naloxone pretreatment. The antinociceptive effects of i.cist. morphine and neurotropin were observed 2 and 5 min after administrations and thereafter every 5 min to 30 min, and the data are shown in Fig. 2.

The antinociceptive effect of morphine at a dose of 0.2 or 0.4 μg/mouse was noted 2 min following i.cist. administration. It reached a maximum and lasted for more than 30 min.
This effect was inhibited by i.p.-preadministered naloxone at 0.5 or 2 mg/kg. The antinociceptive effect of neurotropin, 50 or 100 µg/mouse, peaked 2 min after i.cist. administration, gradually decreased and reached approximately the predrug level after 30 min. This effect in the case of neurotropin was not inhibited by naloxone pretreatment. Naloxone at 0.5 or 2 mg/kg, i.p., had no significant influence on the nociceptive threshold of mice.

A study was then made to determine whether cross-tolerance developed between neurotropin and morphine. The data are shown in Fig. 3.

In non-pretreated mice, the antinociceptive index induced by 10 mg/kg morphine, i.p., was 1.80, but in morphine-tolerant mice, it was 1.28. The antinociceptive index induced by 200 mg/kg neurotropin, i.p., was 1.26.
Fig. 3. The antinociceptive effect of neurotropin in morphine-tolerant mice. Morphine-treated mice had received morphine at 10 mg/kg/day × 3 before the experiments. The pre-drug nociceptive threshold was 101.8±0.5 g (mean±S.E.) in non-treated mice and 99.8±0.7 g in morphine-tolerant mice (not significantly different). No. of animals: 4 mice/group. ***P<0.001, compared with the non-treated group (Student's t-test).

Table 1. Antinociceptive effects of neurotropin on mice in combination with some drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
<th>Antinociceptive index (Mean±S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotropin</td>
<td>150</td>
<td>i.p. 1.21±0.01</td>
</tr>
<tr>
<td>Neurotropin</td>
<td>100</td>
<td>i.cist. 1.40±0.01</td>
</tr>
<tr>
<td>Neurotropin</td>
<td>80</td>
<td>i.th. 1.29±0.02</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>1mg/kg</td>
<td>+Neurotropin 1.17±0.02</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>10mg/kg</td>
<td>+Neurotropin 1.09±0.01**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>20mg/kg</td>
<td>+Neurotropin 1.03±0.01**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>0.2μg/A</td>
<td>+Neurotropin 1.17±0.02**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>0.5μg/A</td>
<td>+Neurotropin 1.37±0.02**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>1μg/A</td>
<td>+Neurotropin 1.24±0.01**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>2μg/A</td>
<td>+Neurotropin 1.19±0.01**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>4μg/A</td>
<td>+Neurotropin 1.08±0.02**</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>8μg/A</td>
<td>+Neurotropin 1.01±0.01**</td>
</tr>
<tr>
<td>Reserpine</td>
<td>0.2mg/kg</td>
<td>+Neurotropin 1.16±0.01*</td>
</tr>
<tr>
<td>Reserpine</td>
<td>1.25mg/kg</td>
<td>+Neurotropin 1.15±0.01**</td>
</tr>
<tr>
<td>Reserpine</td>
<td>2.5mg/kg</td>
<td>+Neurotropin 1.05±0.01**</td>
</tr>
<tr>
<td>Atropine</td>
<td>10mg/kg</td>
<td>+Neurotropin 1.21±0.01</td>
</tr>
<tr>
<td>GABA</td>
<td>1000mg/kg</td>
<td>+Neurotropin 1.17±0.02</td>
</tr>
<tr>
<td>GABA</td>
<td>0.05μg/A</td>
<td>+Neurotropin 1.62±0.02**</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>0.025μg/A</td>
<td>methiodide +Neurotropin 1.14±0.02**</td>
</tr>
<tr>
<td>Aminoxyacetic acid</td>
<td>20mg/kg</td>
<td>+Neurotropin 1.57±0.02**</td>
</tr>
<tr>
<td>Diaminobutyric acid</td>
<td>50mg/kg</td>
<td>+Neurotropin 1.56±0.01**</td>
</tr>
</tbody>
</table>

Phentolamine, GABA and bicuculline methiodide were administered simultaneously with neurotropin by the same route, reserpine was i.p. administered 23 hr before i.p. neurotropin, and atropine, aminoxyacetic acid and dianinobutyric acid were i.p. administered 1 hr before neurotropin. No. of animals: 5–7 mice/group. The pre-drug nociceptive threshold of all mice was 101.1±0.3 g (mean±S.E.). *P<0.05 and **P<0.01, compared with neurotropin alone (Newman-Keuls test).
(P<0.001 vs. predrug value) in non-pre-
treated mice, and in morphine-tolerant mice,
it was 1.25 (P<0.001 vs. pre-neurotropin value),
nearly equal to the level in non-
pre-treated mice. The antinociceptive effect of
neurotropin did not decrease in morphine-
tolerant mice, while it did so in the case of
morphine. There was no cross-tolerance
between neurotropin and morphine.

3. Influence of various drugs on antinocicep-
tive effects of neurotropin

The antinociceptive effects of neurotropin
used in combination with other drugs are
shown in Table 1.

Phentolamine, a noradrenergic blocker,
was administered simultaneously with neuro-
tropin by the same route, and it was found to
inhibit the antinociceptive effect of neuro-
tropin by all modes of administration. Reser-
pine, a depletor of catecholamines, which
was i.p. administered 23 hr before neuro-
tropin, also inhibited the antinociceptive
effect of neurotropin, but atropine, a cholin-
ergic blocker, which was i.p. administered
1 hr before neurotropin, failed to do so.
Atropine doses larger than 10 mg/kg were
not examined, because mice showed slightly
stimulated behavior with a large dose (20
mg/kg) of atropine.

Next, the influence of GABAergic drugs
on neurotropin was examined. GABA had no
influence by the i.p. route, but by the i.cist.
route, it enhanced the antinociceptive effect
of neurotropin. Bicuculline methiodide, a
GABA<sub>A</sub>-antagonist, significantly inhibited
the effect of neurotropin. Aminooxyacetic acid, a
GABA-transaminase inhibitor, or diamino-
butyric acid, a GABA-reuptake inhibitor, sig-
nificantly strengthened the effect of i.cist.
neurotropin. In these cases, GABA and
bicuculline methiodide were administered
simultaneously with neurotropin by the same
route, and aminooxyacetic acid and diamino-
butyric acid were i.p. administered 1 hr
before neurotropin.

All these drugs except for neurotropin
were administered at a dose that would not
directly influence the nociceptive threshold
in mice at the test time.

4. The antinociceptive effect of combined
use of neurotropin and GABA agonists

i.p. administration: GABA agonists were
i.p. administered simultaneously with neuro-
tropin. These effects of neurotropin in combi-
nation with GABA agonists at fixed doses are
shown in the left panel of Fig. 4, and those of
GABA agonists in combination with neuro-
tropin at fixed doses are shown on the right
side.

Two-way ANOVA showed no significant
interaction among the treatments shown on
panels A, B, C and D in Fig. 4, and signi-
ficant interactions were observed among
the treatments with neurotropin and baclofen
shown in panels E (P<0.01) and F (P<
0.001) in Fig. 4. From the results of these
analyses and the regression lines in Fig. 4,
the combined use of neurotropin and GABA
showed the same antinociceptive effects as
those due to neurotropin alone. The combined
use of neurotropin and muscimol, a GABA<sub>A</sub>
agonist, showed additive effects, as shown in
panels C and D. With the combined use of
neurotropin and baclofen, a GABA<sub>B</sub> agonist,
ANOVA revealed a highly significant interaction ($\text{P}<0.01$ in F and $\text{P}<0.001$ in G), and the effects were additive at lower doses of baclofen, whereas at higher doses, no such effects were observed.

**i.cist. administration:** Neurotropin was i.cist. administered simultaneously with GABA agonists, and the data shown in Fig. 5 in the same manner as for Fig. 4. The combined use of neurotropin and GABA showed additive antinociceptive effects, and the combination of neurotropin and muscimol also did so, as in the case of the i.p. route. ANOVA showed a highly significant ($\text{P}<0.001$) interaction for the simultaneous administration of neurotropin and baclofen, and the two drugs were antagonistic at higher doses of baclofen.

**i.th. administration:** Neurotropin was i.th. administered simultaneously with baclofen, and these data are shown in Fig. 6. ANOVA showed significant differences between the treatments shown in the left panel ($\text{P}<0.001$) and the right panel ($\text{P}<0.05$) in Fig. 6. For combined use of neurotropin and baclofen by the i.th. route, no additive effect was observed at higher doses of baclofen, as was also noted for administration by the i.p. and i.cist. routes.

### 5. Combined use of neurotropin and bicuculline methiodide

Figure 7 shows the effects of muscimol and neurotropin co-administered intracisernally with bicuculline methiodide. The antinociceptive effects of muscimol were antagonized by bicuculline methiodide, 0.025 $\mu$g/mouse, and the dose-response curve showed a parallel down-shift. The antinociceptive effect of neurotropin was also antagonized by bicuculline methiodide, 0.025 $\mu$g/mouse, in a similar manner, with the
**Fig. 7.** The antagonistic effect of bicuculline methiodide on antinociception by i.cist. muscimol or neurotropin in mice. Bicuculline methiodide was simultaneously administered with muscimol or neurotropin. Data are shown in the same manner as for Fig. 4. No. of animals: 4 mice/group. The pre-drug nociceptive threshold of these mice was 99.6±0.2 g (mean±S.E.).

dose-response curve also showing the same shift.

**Discussion**

Neurotropin has been reported to show a larger antinociceptive effect on SART-stressed mice than on non-stressed mice (6); and clinically, it has been effective on pain in patients with cervicodynia, post-herpetic neuralgia and other neuropathies. In this study, neurotropin administered by all of the i.p., i.cist. and i.th. routes, showed a larger effect on SART-stressed mice, a model animal with chronic pain (16), than on non-stressed mice. Thus, the antinociceptive effect of neurotropin was larger in diseased mice compared with its effect in normal healthy mice. This fact may be related to findings that SART-stressed animals were in the state of reduced sympathetic tone (8, 11), that some of the above-described pain on which neurotropin shows analgesic effects, are thought to have some relation to the sympathetic nervous system (17–19), and that neurotropin had regulative actions on abnormal tone in autonomic nerves (20, 21).

Effects of neurotropin produced by the i.cist. route were larger than those produced by the i.th. route, so that it appeared to act at the supraspinal level rather than in the spinal cord.

This study attempted to reveal part of the antinociceptive mechanism of neurotropin in normal mice, mainly in relation to opioids, noradrenergic and GABAergic drugs, although it will also be necessary to examine its relation to other pain inhibitory systems, pain transmitters and other factors.

The antinociceptive effect of neurotropin is thought to be non-opioid in nature, because it was not blocked by the opiate antagonist naloxone, and no cross-tolerance developed between neurotropin and morphine.

The antinociceptive effect of neurotropin was blocked by phentolamine and reserpine, but not by atropine. It thus appears that the antinociceptive action of neurotropin may be mediated by a descending noradrenergic system, but unrelated to a cholinergic system.

The antinociceptive effect of neurotropin was enhanced by aminoxyacetic acid and diaminobutyric acid, which activate the GABAergic neuron by inhibiting GABA transaminase or GABA reuptake, and it was antagonized by bicuculline methiodide, a GABA<sub>A</sub> antagonist. Moreover, the combined use of neurotropin with GABA (i.cist.) or muscimol (i.p., i.cist.) exhibited additive effects, but combined use with baclofen did not do so. Thus, the action of neurotropin may be mediated by a GABAergic system, particularly the GABA<sub>A</sub> system.

With regard to its relation with antinociception, GABA is known to be correlated with the action of morphine (22–27). The antinociceptive effect of morphine has been found to be enhanced by aminoxyacetic acid, but lessened by bicuculline (22). Also, GABA content is known to increase significantly with the antinociceptive action of morphine (23, 24) in the dorsal part of the spinal cord, the ventrolateral part in the ventral thalamic nucleus and the lateral...
spinothalamic tract. It is thus likely that GABA plays a part in nociceptive inhibition in these areas. Although it should be determined whether neurotropin increases GABA content, GABA is certainly related to the antinociceptive action of neurotropin.

Neurotropin has also been reported to inhibit the release of bradykinin-like substances induced by noxious stimuli applied to the rat hind paw (28), and it has been observed to inhibit both the first phase involving substance P and bradykinin and the second phase involving histamine and prostaglandin in hyperalgesic mice in the formalin test (12).

In summary, neurotropin may exert analgesic effects both by acting on the central pain inhibitory system and directly and/or indirectly inhibiting the release of pain transmitters.

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