Analgesic Mechanism of Neurotropin: Relation to the Serotonergic System and Influence of Spinal Cord Transection

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Abstract—Neurotropin, a nonprotein component extracted from the skin of rabbits treated with vaccinia virus, has been clinically and experimentally reported to demonstrate analgesic effects. In this study, we investigated the antinociceptive action of neurotropin in relation to the serotonergic system, a pain inhibitory system, and substance P, a pain transmitter; we also attempted to determine whether it acts at the spinal or supraspinal level in mice. 1) The spinal cord (T_6 – T_{10}) transection completely abolished the antinociceptive action of neurotropin, attenuated that of morphine, and had no influence on the action of clonidine. 2) The intrathecal substance P-induced behavior was inhibited by [D-Pro², D-Trp⁷⁻⁹]-substance P, but not by neurotropin. 3) Preadministration of p-chlorophenylalanine or cyproheptadine inhibited the antinociceptive action of neurotropin. These data suggest that neurotropin does not directly act on pain transmitters at the spinal cord level, but acts at the supraspinal level, resulting in an inhibition of pain transmitter release at the spinal level by mediating pain inhibitory systems such as the serotonergic system in addition to the noradrenergic and GABAergic systems previously reported.

Neurotropin, which is a nonprotein component extracted from the skin of rabbits treated with vaccinia virus, has been reported to show a more pronounced antinociceptive effect on diseased animals than on normal healthy animals, and it exerts strong antinociceptive effects on various types of hyperalgesic animals (1–3). Clinically, it is effectively used to treat severe pain in patients with lumbago, cervicodynia, SMON and various neuropathies (4–7). The analgesic effect of neurotropin on orthopedic pain is suggested to result, according to thermographic evaluation, from the improved peripheral blood flow (6). Also, we previously reported that the antinociceptive action of neurotropin differs in degree according to the administration route, and that its action appears to be non-opioid, and may be mediated by the noradrenergic and GABAergic systems (3).

In this report, we investigated whether the antinociceptive action of neurotropin originated in the spinal or supraspinal sites or both.

In addition, we examined whether neurotropin had a direct antagonistic action against substance P, a neurotransmitter for primary nociceptive afferent fibers, by observing the antagonistic action against the behavioral response in mice induced by intrathecal substance P (8, 9).

Next, we studied the antinociceptive action of neurotropin in relation to the serotonergic system, an important pain inhibitory system. We determined its effects by both the tail pressure and tail flick methods; this is because the system was reported to be very related to thermal stimuli (10).

Materials and Methods

Animals: Male ddY mice (Japan SLC, Inc., Shizuoka) weighing about 25 g were used.

Drug administration: Drugs were administered intracisternally, intrathecally or intraperitoneally.

Intracisternal (i.cist.) injection was carried out using a J-shaped needle (27 gauge) curved 40° at 3.5 mm from the tip, according

to the method of Ueda et al. (11). Each mouse was held by hand for this purpose. The needle was inserted into the cleft between the occiput and atlas vertebra through the intact skin. The injection volume was 10 μ l.

Intrathecal (i.th.) injection was carried out by lumbar puncture, introducing the needle (30 gauge) into an intervertebral space approximately at the level of the 5th or 6th lumbar vertebrae, according to the method of Hylden and Wilcox (12). The drug solution was injected at a volume of 5 μ l.

Determination of antinociceptive effects: Nociceptive threshold was assessed by the tail-pressure and tail-flick methods.

The mechanical nociceptive threshold of mice was determined by the modified tail-pressure method using a Randall-Selitto Analgesy-Meter (Ugo Basile). The force was applied to the tail at a point 1 cm distal from the root and increased at a constant rate of 16 g/sec. The force required to produce such reaction in the mouse as lifting, dropping or flexing its tail was defined as the nociceptive threshold.

The thermal nociceptive threshold of mice was quantified by the radiant heat method of D'Amour-Smith (13). Mice were subjected to the radiant heat of a 250 W tungsten lamp at the tip of their tail. The nociceptive threshold was defined as the latency (sec) of tail flexion. The lamp voltage was set to produce a pre-treatment latency of approximately 10 sec.

These two types of methods were applied to both intact mice and spinal transected mice to determine the nociceptive threshold.

The antinociceptive effect of drugs was expressed as the antinociceptive index, which is the ratio of the nociceptive threshold after treatment to that before treatment. The antinociceptive action of neurotrophin was observed twice at 45 and 60 min after i.p., 2 and 5 min after i.cist., and 5 and 8 min after i.th. administration; that of morphine and clonidine was observed twice at 30 and 45 min and at 15 and 30 min after the i.p. administration, respectively. In all cases, the mean of the two values were used as data.

Spinal cord (T₆–T₁₀) transection: The mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Abbott, Nembutal®), and the spinal cord was severed at a point between

the 6th and 10th thoracic vertebrae with a sharp safety razor according to the method of Zorn and Enna (14). Nociceptive threshold was examined 3 days after surgery.

Behavioral action of substance P: The action of substance P on mouse behavior was observed according to the method described by Hylden and Wilcox (9). The intensity of the action was quantified according to the total number of observed behaviors, consisting of bitings, lickings and scratchings of the abdomen and hind portion of the body, during a 2-min counting session from immediately after the injection, and the dose of substance P used was 30 ng/mouse, in accordance with our previous report (15).

Drugs: Neurotrophin® (Nippon Zoki, 20 mg/ml), p-chlorophenylalanine methyl ester hydrochloride (PCPA, Sigma), cyproheptadine (Sigma), clonidine hydrochloride (Sigma), morphine hydrochloride (Takeda), substance P (Peptide Institute Inc.) and [D-Pro², D-Trp^{7,9}]-substance P (Peptide Institute Inc.) were used, and these drugs were dissolved or diluted in 0.9% saline or pure water.

Results

1. Antinociceptive action of neurotrophin in spinal transected mice: The antinociceptive actions of some i.p. administered drugs were observed in intact or spinal cord transected mice. These data are shown in Fig. 1. Clonidine showed similar antinociceptive action in spinal cord transected mice and in intact mice. The antinociceptive action of morphine was markedly attenuated by spinal cord transection, but its action was observed to remain dose-dependent. The antinociceptive action of neurotrophin was abolished completely by spinal cord transection. These results were much the same in the tail-pressure method and in the tail-flick method.

2. No relation of neurotrophin to substance P-induced behaviors was noted: Table 1 shows the total counts of behavioral responses induced by i.th. administration of 30 ng substance P/mouse with and without neurotrophin or [D-Pro², D-Trp^{7,9}]-substance P. When substance P was coadministered with neurotrophin, the count was much the same as the control count. However, substance P-induced behavior was inhibited by

[D-Pro², D-Trp^{7,9}]-substance P, a substance P antagonist; and the total count was significantly less than the control value. Neurotropin doses larger than 80 μ g/mouse were not examined in this experiment, because the administration volume was excessive for mice.

3. Influence of PCPA on the antinociceptive action of neurotropin: Figure 2 shows the

Fig. 1. Disappearance of the antinociceptive effect of neurotropin in spinal cord-transected mice. —: Intact mice, ----: transected mice. Nociception was measured 3 days after spinal cord transection. Pre-drug weight causing nociception (mean $\pm$ S.E.) was 101.8 $\pm$ 0.2 g (66 intact mice) or 91.1 $\pm$ 0.3 g (62 transected mice), and pre-drug tail-flick latency (mean $\pm$ S.E.) was 10.31 $\pm$ 0.06 sec (66 intact mice) or 9.14 $\pm$ 0.05 sec (62 transected mice). Each point represents the mean with S.E. value from 4–9 mice. Points only present means with S.E. values smaller than 0.01.

antinociceptive action of neurotropin on the mice pretreated with PCPA, a serotonin depletor. The actions of neurotropin, 150 and 200 mg/kg, were dose-dependently inhibited by 300 and 500 mg/kg of PCPA in both the tail-pressure method and the tail-flick method.

4. Influence of cyproheptadine on the antinociceptive action of neurotropin: Figure 3 shows the antinociceptive action of neurotropin on mice treated with cyproheptadine, a serotonin receptor blocker.

When neurotropin was simultaneously i.p. administered with cyproheptadine, its antinociceptive action decreased in a dose-dependent manner by cyproheptadine. The antinociceptive index of i.cist. or i.th. neurotropin also decreased in a dose-dependent manner by cyproheptadine.

Discussion

In order to establish whether neurotropin acts directly at the spinal level or indirectly through acting at some supraspinal site, its action was examined as compared with the actions of morphine and clonidine in spinal cord (T₆–T₁₀)-transected mice. The spinal transection partially reduced the antinociceptive action of morphine, this in accordance with previous reports (16, 17) that morphine induces its antinociceptive action by acting at both supraspinal and spinal sites. The action of clonidine was not inhibited by the transection, in agreement with a previous report (18) that it induces an antinociceptive action by acting mostly at a spinal site. These data agree with the results of other investigators (14, 19, 20) as well. On the other hand, the antinociceptive action of neurotropin was completely abolished by the spinal cord transection between T₆ and T₁₀. This fact suggests that

Table 1. Influence of neurotropin on substance P-induced behavior in mice

Drug		Response/2 min (count)
Substance P		95.6 $\pm$ 2.7
Substance P + Neurotropin	40 μ g/mouse	90.8 $\pm$ 7.4
Substance P + Neurotropin	80 μ g/mouse	99.7 $\pm$ 2.4
Substance P + DPDT	800 ng/mouse	51.5 $\pm$ 5.4**

DPDT: [D-Pro², D-Trp^{7,9}]-substance P. The response was quantified as the total number of observed lickings, bitings and scratchings. Neurotropin or DPDT was intrathecally coadministered with 30 ng substance P/mouse. Each value represents the mean with S.E. from 6 or 7 mice. **P<0.01, from the group of substance P only (Newman-Keuls' test).

Fig. 2. Decrease in the antinociceptive effect of neurotropin (NTP) caused by p-chlorophenylalanine (PCPA). Antinociceptive index was the mean of two values determined at 45 and 60 min after i.p. NTP. PCPA was i.p. administered 23 hr before NTP. Weight causing nociception before NTP (mean±S.E.) was 102.2 ± 0.4 g (non-treatment), 101.7 ± 0.5 g (23 hr after 300 mg/kg of PCPA) or 100.8 ± 0.2 g (23 hr after 500 mg/kg of PCPA). Tail flick latency (mean±S.E.) before NTP was 10.48 ± 0.04 sec (non-treatment), 9.78 ± 0.07 sec (23 hr after 300 mg/kg of PCPA) or 9.44 ± 0.09 sec (23 hr after 500 mg/kg of PCPA). No. of mice: 4–6/group. ** $P < 0.01$, from the respective control group treated with NTP only (Newman-Keuls' test).

Fig. 3. Decrease in the antinociceptive action of neurotropin (NTP) caused by cyproheptadine (CYP). CYP was i.p. administered simultaneously with i.p. NTP or 10 min before i.cist. or i.th. NTP. Pre-drug weight causing nociception was 101.7 ± 0.1 g ($n=74$), and pre-drug tail-flick latency was 10.39 ± 0.02 sec ($n=72$). No. of mice: 5–8/group. ** $P < 0.01$, from the respective control group treated with NTP only (Newman-Keuls' test).

neurotropin administered systemically exhibits its antinociceptive action by acting principally at a supraspinal site rostral to T_6 – T_{10} .

In addition, neurotropin administered by the i.th. route also exhibited the antinociceptive action (Fig. 3). From this fact, it is thought that neurotropin may be able to directly inhibit the release of pain transmitters at the spinal site, if only it is able to reach the site. When neurotropin is systemically administered, however, it may be kept from reaching the spinal site by some barrier.

Neurotropin had no influence on i.th. sub-

stance P-induced behavioral response, which is accepted to be the result of noxious stimuli caused by substance P (8, 9). Namely, neurotropin exhibited the antinociceptive action on the nociception induced by the mechanical noxious stimuli, which were reported to cause release of substance P in the spinal cord (21), but had no influence on the behavioral response induced by exogenous substance P at the spinal level. In other words, neurotropin may not have a direct inhibitory action on substance P or substance P receptors in the spinal cord.

Drugs	Route	Effect of neurotropin		
		i.p.	i.cist.	i.th.
Phentolamine	i.p.	↓		
	i.cist.		↓	
	i.th.			↓
Reserpine	i.p.	↓		
Cyproheptadine	i.p.	↓	↓	↓
p-Chlorophenylalanine	i.p.	↓		
Atropine	i.p.	↔	↔	
Bicuculline	i.cist.		↓	
Naloxone	i.p.		↔	

Fig. 4. A schema of influences of various drugs on the antinociceptive effect of neurotropin. ↓ or ↔ indicates decrease in the antinociceptive effect of neurotropin or no influence. Some of these data were quoted from Hata et al., Ref. No. 3.

The antinociceptive action of neurotropin was inhibited by PCPA, a serotonin depletor, and cyproheptadine, a serotonergic receptor blocker. From this, the action of neurotropin is thought to be related to the serotonergic system. The serotonergic system in pain inhibitory systems is reported to be more deeply related to thermal stimuli than to mechanical stimuli (10), but neurotropin showed antinociceptive action to a similar degree in the tail-pressure test, based on mechanical stimuli as well as in the tail-flick test, based on thermal stimuli; and its effect was similarly inhibited by PCPA or cyproheptadine in both tests. Furthermore, the effect of neurotropin administered by all of the i.p., i.cist. and i.th. routes was similarly inhibited by cyproheptadine. Then the antinociceptive action of neurotropin is thought to be related to the serotonergic system at either site of the supraspinal or spinal cord, so long as it reaches the site.

The influences of various drugs used in previous (3) and present studies on the antinociceptive action of neurotropin are summarized in Fig. 4. The antinociceptive action of neurotropine was inhibited by phentolamine, an α -blocker; reserpine, a catecholamine depletor; cyproheptadine, a serotonin receptor blocker; PCPA, a serotonin depletor; and bicuculline, a GABA_A antagonist; and not influenced by atropine, a muscarinic acetylcholine receptor blocker, or naloxone, an opiate antagonist. These data suggest that

the antinociceptive action of neurotropin may be related to all of the serotonergic, noradrenergic and GABAergic systems, rather than to only one of these systems.

In summary, it is suggested that neurotropin has no direct inhibitory action on the action or receptor of pain transmitter at the spinal cord, but instead that it, administered systemically, may act at some supraspinal site, resulting in an inhibition of the release of pain transmitters at the spinal site by mediating descending pain inhibitory systems such as the serotonergic, noradrenergic and GABAergic systems. However, it should still be studied how these systems interact on the action of neurotropin and whether it acts on diseased animals such as some hyperalgesic or chronic-stressed animals via the same mechanism.

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