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Structure-antinociceptive activity studies with neurotensin

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1 The antinociceptive effects of synthetic neurotensin (NT), its fragments and analogues administered into the lateral cerebroventricle have been compared in the conscious mouse.

2 Intracerebroventricular (i.c.v.) administration of NT produced a dose-dependent antinociceptive effect in the tail pressure test.

3 The NT fragments and analogues, NT(8–13), NT(8–10), NT(9–13), NT(9–11), NT(8–11) NHEt and NT(9–11) NHEt were also effective antinociceptive peptides.

4 The potency of NT(8–13) and the duration of its effects were found to be approximately equal to those of NT.

5 The antinociceptive effects produced by NT, NT(8–13) and NT(9–13) were significantly reversed by the opioid antagonist naloxone but not by thyrotropin releasing hormone.

6 It is concluded that NT(8–13) is required for the full expression of the antinociceptive effects of NT which may be mediated in part through the brain opioid system.

Introduction

Neurotensin (NT), an endogenous tridecapeptide (*p*-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) which was originally isolated from extracts of bovine hypothalami (Carraway & Leeman, 1973), has a wide range of biological activities. Exogenously administered tridecapeptide exerts a variety of behavioural effects, including antinociception and hypothermia (for review, see Leeman & Carraway, 1977; Bissette *et al.*, 1978). NT-induced antinociception was first described by Clineschmidt & McGuffin (1977) who showed a potent and long-lasting increase in the pain threshold; the antinociceptive activity of NT was not antagonized by naloxone, an opioid antagonist (Nemeroff *et al.*, 1979). There is conflicting evidence in the literature regarding the behavioural consequences of intracerebral injection of NT. According to recent reports, NT has naloxone-reversible antinociceptive properties (Kudo *et al.*, 1980; von Wimersma Greidanus *et al.*, 1982). Additionally, it has been found that NT-induced antinociception was antagonized by thyrotropin releasing hormone (TRH) (Osbahr *et al.*, 1981). Structure-activity relationship studies have shown that the biological activity of NT is due to its C-terminal hexapeptide (Carraway & Leeman, 1975; Folkers *et al.*, 1976). There is, however, no available publication describing the antinociceptive action of NT fragments or analogues.

The present study was performed with two aims in mind. The first was to determine the important part within the structure of NT in producing antinociception. The second was to re-evaluate earlier observations on the effects of naloxone or TRH on NT-induced antinociception utilizing the tail-pressure test in mice.

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Methods

Mice (ddY-male) weighing 22–25 g were used in all experiments. They were supplied with food and water *ad libitum* and kept on a 12 hour light-dark cycle. They were housed at least two days before their use and were used only once. Groups of ten mice were used for each experiment. Antinociceptive activities were determined using tail pressure as previ-

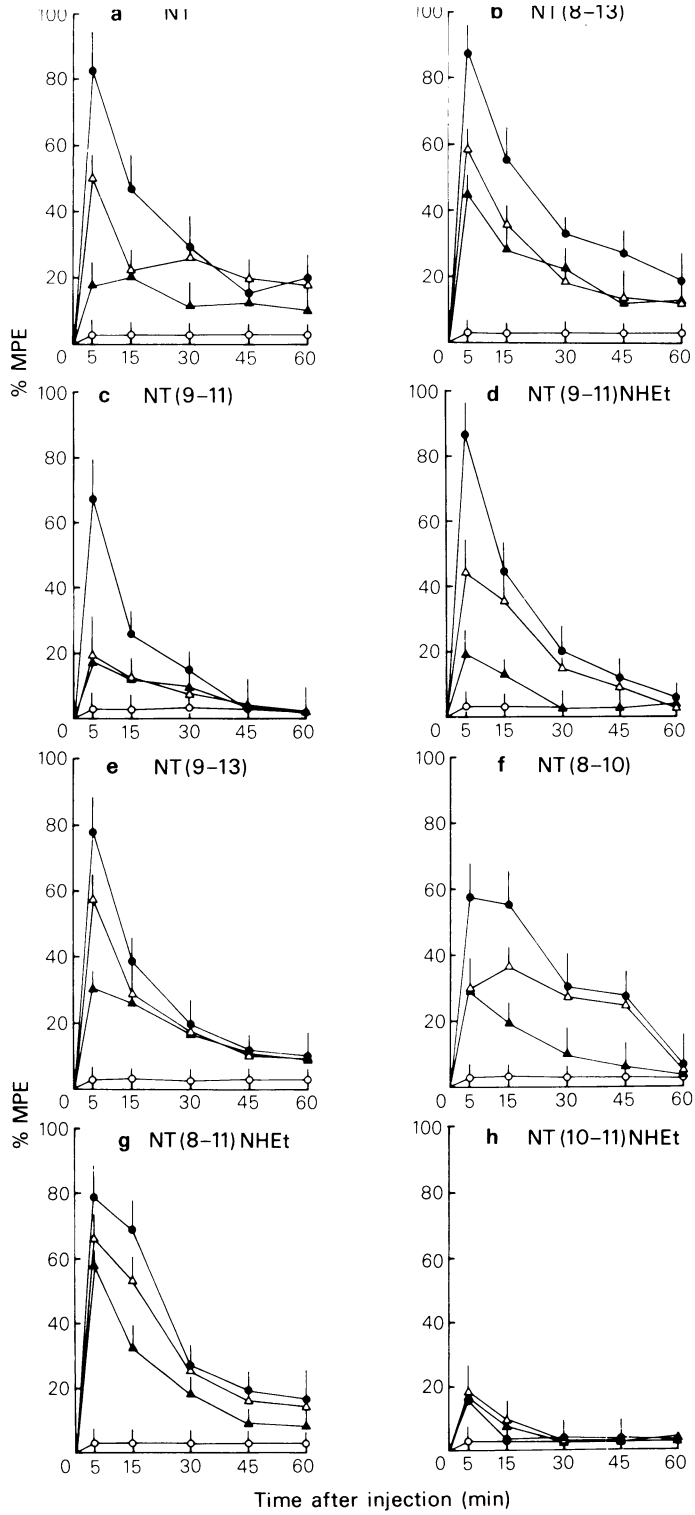

Figure 1 The antinociceptive effect and time course of neurotensin (NT), its analogues and fragments administered intracerebroventricularly in mice. (a) NT, 28.6 (▲), 48.3 (△) and 62.7 (●) nmol. (b) NT(8–13), 48.3 (▲), 81.6 (△) and 137.9 (●) nmol. (c) NT(9–11), 62.7 (▲), 137.9 (△) and 302.9 (●) nmol. (d) NT(9–11) NHEt, 62.7 (▲), 106.0 (△) and 179.2 (●) nmol. (e) NT(9–13), 106.0 (▲), 393.7 (△) and 665.4 (●) nmol. (f) NT(8–10), 62.7 (▲), 106.0 (△) and 179.2 (●) nmol. (g) NT(8–11) NHEt, 81.6 (▲), 106.0 (△) and 137.9 (●) nmol. (h) NT(10–11), 179.2 (▲), 393.7 (△) and 511.9 (●) nmol. In (a–h), (○) represents control.
Each point represents the mean ± s.e.mean (vertical lines) of ten mice in each group. Control groups were treated with Ringer.

ously described (Sakurada *et al.*, 1982). The base of the tail was pressed mechanically (10 mmHg s^{-1}) and the level of pressure in mmHg that evoked biting or an aversive response was noted. Only mice responding behaviourally to a tail pressure of 40–50 mmHg were selected for this test. The responsive pressure before drug injection was 4.4 ± 0.2 mmHg ($n = 100$). A value of 100 mmHg was used as the cut-off pressure to avoid tail tissue damage. The antinociceptive activity for each mouse was calculated according to the following formula:

$$\% \text{ of antinociception} = (P_2 - P_1/100 - P_1) \times 100$$

where P_1 is the responsive pressure before drug injection (mmHg) and P_2 is the responsive pressure after drug injection (mmHg). The data are expressed as mean % of antinociception ± s.e. At 5, 15, 30 and 60 min following injection, tail pressure thresholds were determined. Mice were injected i.c.v. with Ringer solution or peptides dissolved in Ringer solution at various time intervals before being tested. The technique employed for i.c.v. injection has been described elsewhere (Orikasa *et al.*, 1980).

The following drugs were used: NT (Protein Research Foundation, Osaka), NT(8–13), NT(8–10), NT(9–13), NT(9–11), NT(10–11) NHEt, NT(8–11) NHEt, NT(9–11) NHEt, naloxone (Endo Laboratories) and TRH (Protein Research Foundation, Osaka). NT fragments and analogues were synthesized in our laboratory by the conventional liquid phase method. Physiochemical properties of these NT fragments and analogues are listed in Table 1. In all experiments, naloxone (0.1, 1.0 or 2.0 mg kg^{-1}) was injected intraperitoneally 20 min before peptide administration. TRH (4.0, 8.0 or 16.0 nmol per animal) was co-administered i.c.v. with NT, NT(8–13), or NT(9–13). A minimum of 3 doses (10 mice per dose) was used in all cases. ED_{50}, 95% confidence limits and significance of difference in potency were determined according to the method of Litchfield & Wilcoxon (1949). The statistical significance of the results was calculated using Student's *t* test for paired data and *P* values of 0.05 or less were considered significant.

Results

Antinociceptive effects of neurotensin (NT) and its fragments or analogues

The time courses of the antinociceptive effects after injection of various doses of NT, NT(8–13), NT(8–10), NT(9–13), NT(9–11), NT(8–11) NHEt, NT(9–11) NHEt and NT(10–11) NHEt are shown in Figure 1. I.c.v. injection of NT caused a dose-related antinociceptive effect in doses ranging from 28.6 to 62.7 nmol per mouse. NT gave a peak antinociceptive activity at 5 min falling away at 60 min at the maximally effective dose of 62.7 nmol per mouse. The effective antinociceptive dose, ED_{50} (95% confidence limits) was 49.0 (40.0–59.2) nmol per mouse with the tail pressure test (Table 1). Administration of Ringer solution did not induce significant effects. From the ED_{50} values, NT was 3.5 times more potent than Met-enkephalin which gave a peak of short duration 2 min after injection (Sakurada *et al.*, 1984). Two synthetic peptides, having the sequence of C-terminal stretches of NT, were also tested for antinociceptive activity. NT C-terminal

Table 1 Antinociceptive activities produced by intracerebroventricular administration of neurotensin (NT) and its analogues measured using the mouse tail pressure test

Compound	$[\alpha]_D^{20}$ [a]	t.l.c.[b]	*Peak time* (min)	*ED_{50}* (nmol per mouse)[c]	*Relative potency*[d]
NT	—	—	5	49.0 (40.5–59.3)	1.0
NT(8–13)	−52.40	0.18	5	50.0 (34.0–73.5)	0.98
NT(9–13)	−58.95	0.23	5	84.0 (36.2–194.9)	0.58
NT(8–10)	−25.74	0.05	5	110.0 (58.2–207.9)*	0.45
NT(9–11)	−22.90	0.14	5	230.2 (158.6–333.5)*	0.21
NT(9–11) NHEt	−36.20	0.17	5	110.0 (65.8–185.9)*	0.45
NT(8–11) NHEt	−36.44	0.12	5	68.0 (42.0–110.2)	0.72
NT(10–11) NHEt	− 3.80	0.36	5	>400.0	—

[a] Optical rotation measured with a JASCO DIP-140 polarimeter, at a concentration c = 1 in water.
[b] Thin layer chromatography on silica gel plates $60F_{254}$ (Merck) in the solvent system: *n*-butanol:acetic acid:water (4:1:5, upper phase).
[c] ED_{50} values were calculated from the values obtained at the time of peak effect, 95% confidence limits are given in parentheses.
[d] Potencies are relative to neurotensin (NT) (= 1.0) on a molar basis.
* The ED_{50} value was significantly less than that obtained with NT ($P < 0.05$).

Table 2 Effects of naloxone on the antinociceptive activities of neurotensin (NT), NT(8–13) and NT(9–13) given intracerebroventricularly in the mouse

Treatments (mg kg^{-1}, s.c.)/(nmol per animal)	*Change in threshold* (%)
Saline/Ringer	2.1 ± 2.0
Saline/NT (62.7)	86.3 ± 6.7
Naloxone (0.1)/NT (62.7)	59.1 ± 9.3*
Naloxone (1.0)/NT (62.7)	32.9 ± 8.7***
Naloxone (2.0)/NT (62.7)	31.8 ± 9.1***
Saline/NT (8–13) (137.9)	87.1 ± 6.7
Naloxone (0.1)/NT (8–13) (137.9)	34.6 ± 11.8***
Naloxone (1.0)/NT (8–13) (137.9)	38.8 ± 10.3***
Naloxone (2.0)/NT (8–13) (137.9)	36.3 ± 12.4***
Saline/NT (9–13) (665.4)	77.2 ± 10.1
Naloxone (0.1)/NT (9–13) (665.4)	42.5 ± 10.0***
Naloxone (1.0)/NT (9–13) (665.4)	30.9 ± 9.7***
Naloxone (2.0)/NT (9–13) (665.4)	19.3 ± 11.0***

Naloxone or saline was given 20 min before NT, NT(8–13) or NT(9–13). Nociceptive responses were determined 30 min before and 5 min after i.c.v. injection of each peptide. *$P < 0.05$, ***$P < 0.001$ when compared to each control (saline plus neurotensin or its fragments) group.

hexapeptide (Arg-Arg-Pro-Tyr-Ile-Leu) showed approximately the same antinociceptive potency as NT and its effects had approximately the same duration. The C-terminal pentapeptide, NT(9–13) appeared to have about 60% the potency of NT or NT(8–13), although there was no significant difference in the ED_{50} values when compared using the paired t test. The ED_{50} values of NT(8–10), NT(9–11) and NT(9–11)NHEt was significantly greater than that obtained with NT. No difference in ED_{50} values between these tripeptides was observed. When NT(8–11)NHEt was compared with NT(9–11) NHEt, the ED_{50} value of the former was not significantly lower than that of the latter. The ED_{50} value of NT(10–11) was not calculated because of its low potency.

Table 3 Effects of thyrotropin releasing hormone on the antinociceptive activities of neurotensin (NT), NT(8–13) and NT(9–13) given intracerebroventricularly in the mouse

Treatments (nmol per animal)	*Changes in threshold* (%)
Ringer/Ringer	1.5 ± 3.1
Ringer/NT (62.7)	86.0 ± 6.9
TRH (4.0)/NT (62.7)	58.5 ± 9.1
TRH/(8.0)/NT (62.7)	65.9 ± 11.5
THR(16.0)/NT (62.7)	61.3 ± 12.9
Ringer/NT(8–13) (137.9)	87.0 ± 7.1
THR(4.0)/NT(8–13) (137.9)	60.6 ± 9.8
THR(8.0)/NT (137.9)	56.1 ± 18.7
THR(16.0)/NT (137.9)	63.4 ± 5.4
Ringer/NT(9–13) (665.4)	77.2 ± 7.5
TRH(4.0)/NT(9–13) (665.4)	54.0 ± 15.2
TRH(8.0)/NT (665.4)	57.9 ± 10.0
TRH(16.0)/NT (665.4)	73.3 ± 11.2

Thyrotropin releasing hormone (TRH) or Ringer was co-administered i.c.v. Nociceptive responses were determined 30 min before and 5 min after simultaneous injection of NT or its fragments with TRH.

Effect of naloxone on neurotensin (NT)-, NT(8–13)-, and NT(9–13)-induced antinociception

NT(62.7 nmol per mouse), NT(8–13) (137.9 nmol per mouse) and NT(9–13) (665.4 nmol per mouse) were administered in doses which significantly elevated the threshold of the tail pressure response. When naloxone, an opioid antagonist (0.1, 1.0 or 2.0 mg kg^{-1}) was injected intraperitoneally 20 min before peptide administration, the antinociceptive effects were antagonized significantly (Table 2).

Effects of thyrotrophin releasing hormone (TRH) on neurotensin (NT)-, NT(8–13) and NT- (9–13)-induced antinociception

When TRH (4.0, 8.0 or 16.0 nmol per mouse) was given at the same time as NT, NT(8–13) or NT(9–13), there was a tendency to reduce the antinociceptive action induced by peptide administration; however, a statistically significant reduction was not observed (Table 3).

Discussion

The results show that i.c.v. injection of NT produced a dose-dependent elevation in nociceptive threshold in mice. All of the fragments or analogues were found to produce a dose-related antinociception similar to that of NT. Of special interest is the finding that NT(9–11)-pentapeptide or NT(8–13)-hexapeptide is necessary at the C-terminal structure to produce the full antinociceptive activity of NT. They also suggest that Arg in position 8 and 9 of the NT C-terminal portion contains chemical groups important for the antinociceptive activity of NT. It was shown by the biochemical studies on the degradation of NT that the main cleavage is at the Arg^8–Arg^9 bond leading to the production of NT(1–8) and NT(9–13) (McDermott *et al.*, 1982). It was also found that the addition of Arg^8 yielded the NT(8–13)-hexapeptide as potent as NT in binding to a cell line (HT 29) derived from human colon carcinoma (Kitabgi *et al.*, 1980). The same phenomonon was demonstrated in the rat isolated heart (Quirion *et al.*, 1980). In the present study, removal of Arg^8 from the C-terminal hexapeptide, NT(8–13), to give NT(9–13) did not produce a significant change in the ED_{50} value. The activity of the tripeptide containing Arg^8, NT(8–10) did not significantly differ from that of NT(9–11)-tripeptide. Thus, the present results provide evidence for the importance of Arg^9 as well as Arg^8 for antinociceptive activity. The binding of NT(8–13) to receptors in brain membranes is reported to be about one-tenth of that for NT (Uhl *et al.*, 1977), although NT(8–13) had the same potency as NT in eliciting antinociception. Hence these results show that the antinociceptive potencies of NT and NT(8–13) are not in parallel with their binding activities. Moreover, the importance of Arg^9 was also suggested by the results of this study; the removal of Arg^9 from NT(9–11) NHC_2H_5 resulted in a marked decrease of antinociceptive potency. As seen by comparison with NT(8–13) or NT(9–13), the addition of ethylamide into NT(8–11) or NT(9–11) may be capable of substituting for the C-terminal two amino acids, Ile and Leu. The two arginine residues at portions 8 and 9 also appear to be extremely important.

The antinociceptive effects of NT, NT(8–13) or NT(9–13) as presently tested by the tail pressure method were counteracted significantly by the opioid antagonist, naloxone. Earlier studies demonstrated the lack of effect of naloxone on NT-induced antinociception (Clineschmidt *et al.*, 1979; Osbahr *et al.*, 1981). However, naloxone has been recently reported to antagonize the antinociceptive action (Kudo *et al.*, 1980; Von Wimersma Greidanus *et al.*, 1982). Thus, although opioid receptors appear to be involved in the antinociceptive effect of NT, it may be that other systems in the CNS concerning antinociception play a role as well. This concept is supported by the present results which showed that NT-induced antinociception was not completely reversed by naloxone ($0.1-2.0\ mg\ kg^{-1}$).

In order to confirm a previous report that both peripherally and centrally administered TRH antagonizes the antinociceptive effects of NT (Osbahr *et al.*, 1981), TRH was simultaneously injected with NT into the cerebroventricle. We have already observed that i.c.v. administration of TRH alone in low doses (4.0–16.0 nmol per mouse), which was the same dose used in the present study, produces weak but significant antinociceptive action, 5 min after the injection, compared to the Ringer control (Kawamura *et al.*, 1984). However, TRH was without significant effect on NT-induced antinociception in the presently described experiments. The differences between the various studies may be explained by differences in the antinociceptive tests and in the doses of NT, as well as in those of naloxone or TRH, used.

In summary, the present data obtained using the C-terminal fragments or analogues of NT emphasize the important contribution of Arg^8 and Arg^9 in the chemical structure of NT for inducing antinociception. Also the results confirm that NT induces antinociception and provide further evidence that this antinociceptive effect may be produced via interaction with opioid receptors.

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