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RAPID AGONIST-INDUCED DECREASE OF NEUROTENSIN RECEPTORS FROM THE CELL SURFACE IN RAT CULTURED NEURONS

MARIE-AGNÈS VANISBERG, JEAN-MARIE MALOTEAUX,* JEAN-NOËL OCTAVE and PIERRE M. LADURON†

Laboratoire de Neurochimie, Université Catholique de Louvain, B-1200 Brussels, Belgium and †Rhône-Poulenc Rorer, Centre de Recherches, 94403 Vitry-sur-Seine, France

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Abstract—The regulation of neurotensin receptors was studied *in vitro* in primary cultures of neuronal cells. High affinity receptors for [^{3}H]neurotensin were found in homogenates and at the cell surface of intact neurons cultured from the brain of rat embryos. When intact cells were incubated with 3 nM neurotensin (1–13), a rapid decrease in [^{3}H]neurotensin binding was observed; about 60% of neurotensin receptors disappeared from the cell surface in less than 15 min. This corresponded to a reduction of the B_{max} value without a change in the binding affinity. The decrease in neurotensin receptor number was also induced by the active fragment (8–13) of neurotensin but not by its inactive fragment (1–8). It was partially inhibited by bacitracin, at concentrations which are known to interact with receptor internalization, and was not detected when intact cells were incubated at 0–4° with the unlabeled peptide. When intact neurons were incubated with [^{3}H]neurotensin, there was a rapid ligand uptake and the kinetics of endocytosis were similar to those of the cell surface receptor disappearance. Once endocytosed, [^{3}H]neurotensin could not be released (or displaced) from either intact neurons or homogenates, suggesting the sequestration of the labeled peptide in vesicles or other subcellular structures. Therefore, the present results suggest that the rapid agonist-induced decrease in the number of neurotensin receptors from the cell surface corresponds to an internalization process which involves a simultaneous receptor-mediated peptide endocytosis.

Neurotensin is a tridecapeptide which was first isolated from bovine hypothalamus [1]. It is present in the digestive tract as well as in the central nervous system of guinea-pig, rat, chicken and human [2–5]. It has a variety of biological activities as a central neurotransmitter or neuromodulator [6], and a peripheral hormone [7].

Neurotensin receptors have been characterized in rat and human brains (for review see Ref. 8). Several reports suggested a single class of high affinity receptors [9, 10] whereas others described two neurotensin binding sites with different affinities [11–13]. Using levocabastine, a potent antihistamine Schotte *et al.* [14] found evidence for two distinct neurotensin binding sites in rat brain: the neurotensin acceptor sites (NT_1 or NT_L sites) which are sensitive to both neurotensin and levocabastine and the neurotensin high affinity receptors (NT_2 or NT_H) sensitive only to neurotensin. Differences in the ontogeny and the regional, and subcellular localizations of NT_1 and NT_2 sites were observed [14–16]. Recently, neurotensin receptors were purified from bovine and mouse brains [17, 18] and the rat neurotensin receptor was cloned [19].

Neurotensin receptors were also studied in various cultured cells: neuroblastoma N1E-115 [20–23], neuroblastoma × glioma NG 108-15 cells [24], primary mice cultured neurons [25] and HT-29 human colonic carcinoma [26]. As a rule, neurotensin receptors were found to be coupled to phosphatidylinositol turnover [27, 28]. However, in neuroblastoma cells, neurotensin receptors have been reported to control the production of cyclic GMP [21].

The regulation of neurotensin receptors was first described in HT-29 cells [29]. In this paper, we present evidence for a rapid neurotensin-induced decrease of the neurotensin receptor number in rat primary cultured neurons.

MATERIALS AND METHODS

Chemicals. Reagents and materials were obtained from the following sources: [^{3}H]neurotensin (1–13) (sp. act.: 65.5, 80.7, 78.3, 83.3, 101.5 Ci/mmol in ethanol) from New England Nuclear (Boston, MA, U.S.A.); neurotensin (1–13), neurotensin (8–13), neurotensin (1–8), bovine serum albumin (BSA‡), methylamine, chloroquine, colchicine and poly-L-lysine from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); EDTA from Merck (Darmstadt, F.R.G.); polyethylenimine and nocodazole from Janssen Pharmaceutica (Beerse, Belgium); bacitracin from Serva (Heidelberg, F.R.G.); 1,10 phenantroline from Aldrich (Steinheim, F.R.G.); tissue culture media and foetal calf serum from Gibco (Paisley, U.K.); tissue culture plastic ware from Falcon, Becton Dickinson (Oxnard, U.S.A.) and Aqualuma from Lumac (Schaesberg, The Netherlands).

* Corresponding author: J. M. Maloteaux, Laboratoire de Neurochimie & Service de Neurologie, Université Catholique de Louvain, 10 Av. Hippocrate, B-1200 Brussels, Belgium. Tel. (32) 2-764-1350.

‡ Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline solution.

Primary cultures of neurons. Embryos from Wistar rats were taken at 16 or 17 days of gestation and dissected under a binocular microscope. The forebrains were retained and the cells were dissociated mechanically with a Pasteur pipette. Cells were plated, at a ratio of one brain per dish, on Petri dishes (60 × 15 mm) which had been coated previously with 10 μg/mL poly-L-lysine.

The culture medium was a mixture of Dulbecco's modified Eagle medium and Ham's F12 (3:1) supplemented with 10% foetal calf serum, 0.6% D-glucose, 100 I.U. penicillin and 100 μg/mL streptomycin. Cultures were maintained at 37° in a humidified atmosphere of 10% CO_2–90% air. After 3 days of culture, 10 μM cytosine arabinoside were added to the medium in order to inhibit non-neuronal cell proliferation and the cells were further incubated. Neuronal cell cultures were used 4, 5, or 10 days after plating as indicated in Results.

When neurons were incubated with neurotensin (1–13) or its fragments, they were maintained for 1 hr at 0–4 or 37° in the culture medium containing the peptide, and control cells were always incubated simultaneously under the same conditions.

Tissue preparation. For the experiments performed with homogenates, cells in culture were rinsed twice with PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) at 0–4° and then resuspended in 50 mM Tris–HCl, pH 7.4.

The cells corresponding to approximately 2 mg of protein were homogenized in 1 mL of cold buffer by 10 up and down strokes in a Dounce homogenizer. The homogenate was centrifuged twice at 48,000 *g* in a Beckman J2 21 for 10 min at 0–4° and maintained at this temperature until the binding assay.

Before the binding assay, proteins were measured and the homogenates were diluted with a 50 mM Tris–HCl, pH 7.4, containing 0.1% BSA, 0.2 mM bacitracin and 1 mM EDTA to obtain a protein concentration of 0.5 mg protein/mL.

The protein level was determined by the method of Lowry *et al.* [30] using BSA as standard.

Binding assay in homogenates. The optimal binding conditions were determined as previously reported using rat brain membranes [14]. The homogenate of neurons—(0.5 mg protein/mL) in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1% BSA, 0.2 mM bacitracin and 1 mM EDTA—was incubated at 25° for 20 min in the presence of [^{3}H]neurotensin (2 nM) with or without 1 μM unlabeled neurotensin (1–13) in order to determine the non-specific binding.

The incubation was terminated by adding 3 mL of cold buffer to the tissue suspension before filtration. The samples were filtered on GF/C Whatman fiber filters soaked previously with 0.5% polyethylenimine for 1 hr and rinsed twice with 3 mL of cold buffer. Filters were counted in plastic vials with 7 mL Aqualuma.

Experiments on homogenates from adult rat brain were performed in the same way. Brain tissue was homogenized with a teflon pestle dual homogenizer and centrifuged twice at 48,000 *g* for 10 min; the pellet was suspended in 10 vol. of 50 mM Tris–HCl, pH 7.4 and incubated for 30 min at 37°. The tissue suspension was then recentrifuged at 48,000 *g* for 10 min and the pellet was finally suspended in 200 vol. of buffer.

The binding conditions and buffer composition were similar to those described for homogenates of cultured cells. At the end of the incubation, the samples were filtered on Whatman GF/B filters presoaked with polyethylenimine.

Binding assay on intact cells. For experiments with intact cells, the culture medium was removed, the cells were washed twice with the culture medium without foetal calf serum and once with binding buffer (50 mM Tris–HCl—250 mM sucrose, pH 7.4, containing 0.1% BSA, 0.2 mM bacitracin and 1 mM EDTA). Binding experiments were performed either at 0–4° or 37°, in Petri dishes with 2 mL of binding buffer. [^{3}H]Neurotensin (2 nM) was incubated for 1 hr with the intact cells in the presence or absence of 1 μM neurotensin (1–13) to determine the non-specific binding. After incubation, the medium was removed and the cells were washed twice with 2 mL of PBS. They were collected rapidly in 0.5 mL of deoxycholic acid, pH 11.3, and counted in plastic vials with Aqua-luma (7 mL) in a Beckman scintillation counter LS 7800.

RESULTS

Characterization of [^{3}H]*neurotensin binding in tissue homogenate and intact neurons*

Homogenates of neurons cultured for 5 or 10 days were incubated with various concentrations of tritiated neurotensin (0.25–8 nM) at 25° for 20 min in the presence or absence of 1 μM unlabeled neurotensin (1–13).

Figure 1 shows a binding experiment performed with different [^{3}H]neurotensin concentrations on homogenates of 5-day-old cultured neurons. Specific binding was saturable. Non-specific binding was low and increased linearly with increasing concentrations of tritiated neurotensin.

The linear Scatchard plot indicates that [^{3}H]-neurotensin was bound to a single class of high affinity sites (Fig. 1). The mean K_D and B_{max} values of three different experiments performed on 5- and 10-day-old cultured neurons are presented in Table 1. [^{3}H]Neurotensin binding was also measured at 0–4° and 37° on intact neurons after 4 days of culture. The Scatchard analysis of the saturation curves revealed K_D and B_{max} values that are in agreement with those obtained on homogenates of neurons (Table 1).

The ability of neurotensin (1–13), neurotensin (1–8) and neurotensin (8–13) to inhibit the binding of [^{3}H]neurotensin was investigated in tissue homogenates and intact neurons.

Homogenates of neurons or intact cells were incubated with [^{3}H]neurotensin (2 nM) at 25° for 20 min (homogenates), or at 0–4° and 37° for 30 min (intact cells) in the presence of increasing concentrations (0.01 nM to 1 μM) of unlabeled peptide.

The IC_{50} values of neurotensin (1–13) and neurotensin (8–13) in [^{3}H]neurotensin binding inhibition experiments performed on homogenates are presented in Table 2.

In intact neurons, the IC_{50} values of neurotensin

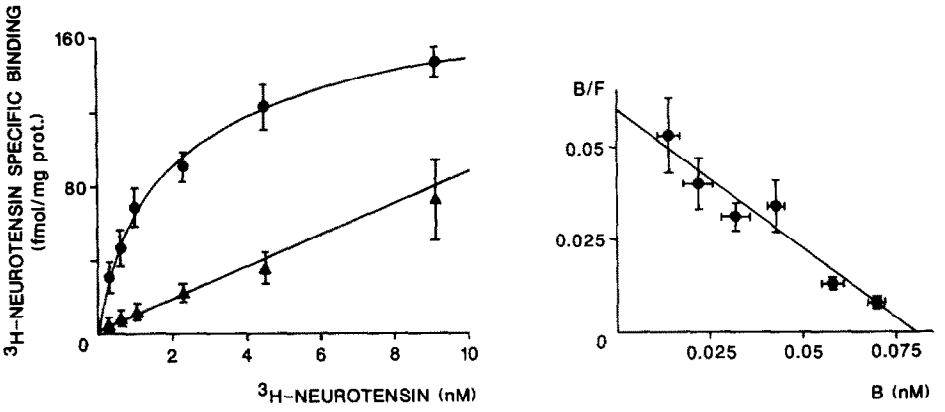

Fig. 1. [^{3}H]Neurotensin specific binding (●) to homogenates of 5-day-old cultured neurons. Non-specific binding (▲) was determined in the presence of 1 μM neurotensin (1–13). Inset: Scatchard analysis of the specific binding. Mean K_D and B_{max} values of three independent experiments performed in triplicate were 1.45 nM and 161.2 fmol/mg protein, respectively. B, concentration of [^{3}H]neurotensin specifically bound (nM); F, concentration of free ligand.

Table 1. K_D and B_{max} values of [^{3}H]neurotensin specific binding in homogenates and intact cultured neurons

		K_D (nM)	B_{max} (fmol/mg protein)
Homogenates from cultured neurons			
Five-day-old culture	25°	1.45 ± 0.47 (3)	161.2 ± 9.7 (3)
Ten-day-old culture	25°	0.98 ± 0.05 (3)	524.1 ± 40.0 (3)
Intact neurons			
Four-day-old culture	0–4°	0.54, 0.90 (2)	80.4, 91.2 (2)
	37°	0.92 ± 0.02 (3)	122.6 ± 44.1 (3)

Results are means ± SD of (N) different experiments. Binding conditions at the indicated temperature are described in Materials and Methods.

Table 2. Binding characteristics of [^{3}H]neurotensin (2 nM) to homogenates and intact neuronal cells (4 days) and in adult rat forebrain

	IC_{50} values (nM)		
	Homogenates from cultured neurons	Intact cells at 0–4°	Rat brain
Neurotensin (1–13)	4.70 ± 2.06 (5)	5.01, 1.99 (2)	7.8 ± 1.5
Neurotensin (8–13)	0.30 ± 0.04 (3)	0.63, 0.25 (2)	0.3 ± 0.1
Neurotensin (1–8)	>10,000	>10,000	>10,000
Levocabastine	>10,000	—	25.0 ±4.0

Results are means ± SD of (N) different experiments performed in triplicate.

(1–13) were 5.01 and 1.99 nM at 0–4°, and 2.81 and 0.71 nM at 37°. Using neurotensin (8–13), the IC_{50} values were 0.63 and 0.25 nM when incubation with intact neurons was performed at 0–4°. The IC_{50} values of neurotensin (1–8) and levocabastine were higher than 10 μM in both intact neurons and their homogenates indicating that, in rat cultured neurons, we were dealing only with high affinity neurotensin receptors.

Association of [^{3}H]*neurotensin*

Kinetics of association of [^{3}H]neurotensin binding were studied at 25° on cell homogenates and at 0–4° or 37° on intact neurons.

On neuronal cell homogenates, the association of [^{3}H]neurotensin to its binding sites was rapid and similar to that observed on adult rat brain homogenates (data not shown).

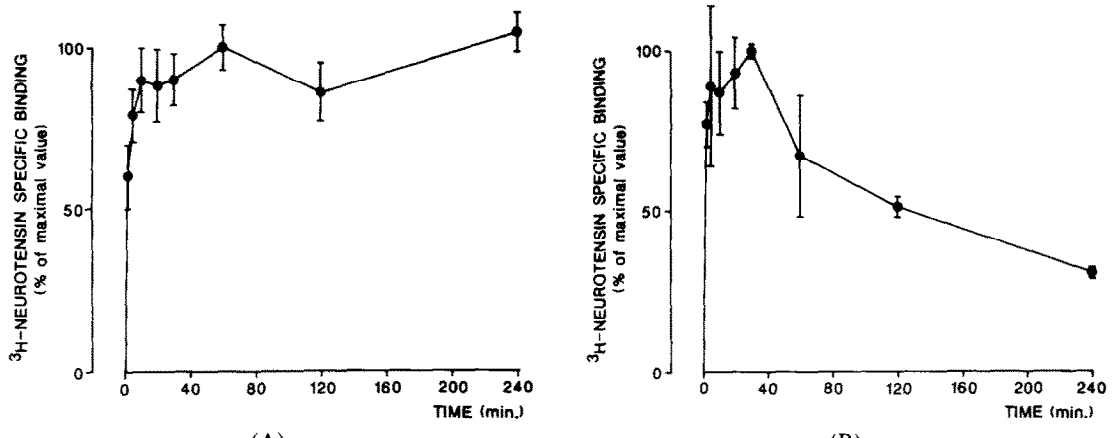

Fig. 2. Kinetics of association of [^{3}H]neurotensin to intact neuronal cells incubated at 0–4°. Association of [^{3}H]neurotensin (2 nM) was measured at 0–4° on intact 5-day-old cultured neurons, in the presence (A) or the absence (B) of bacitracin (0.2 mM). Results are expressed as the means ± SEM of nine different experiments performed in triplicate.

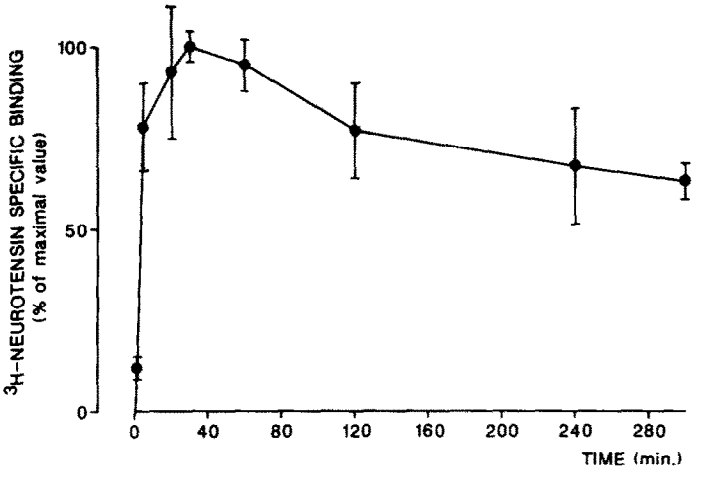

Fig. 3. Kinetics of association of [^{3}H]neurotensin (2 nM) to intact neuronal cells incubated at 37°. Results are expressed as the means ± SEM of experiments performed in triplicate on 5-day-old cultures (N = 11) in the presence of bacitracin (0.2 nM).

[^{3}H]Neurotensin (2 nM) also associated rapidly to its receptors at 0–4° on 5-day-old intact neurons. Maximal association (109.8 ± 33.2 fmol/mg protein) was obtained after 60 min of incubation (Fig. 2A, mean ± SEM of nine different experiments) and remained stable until 240 min when bacitracin (0.2 mM) was present in the incubation medium. In the absence of bacitracin a progressive decrease of the [^{3}H]neurotensin binding to the intact cells was observed (Fig. 2B).

At 37°, in the presence of bacitracin (0.2 mM), the association of [^{3}H]neurotensin (2 nM) to intact neurons was comparable to that measured at 0–4° and corresponded to 169.4 ± 22.4 fmol/mg protein after 30 min (Fig. 3, mean ± SEM of 11 different experiments). The specific binding was 163.9 ± 22.8 fmol/mg protein after 60 min of incubation. When incubation of neurons was performed at 37° for more than 1 hr with the labeled ligand, a progressive decrease of the binding was observed (Fig. 3).

Specific binding was reduced to 63% of its maximal value after 300 min of incubation in the presence of bacitracin (0.2 mM) and this decrease was not prevented by addition of 1,10 phenantroline (1 mM) or colchicine (10 μM).

Dissociation of [^{3}H]*neurotensin binding*

The kinetics of dissociation of [^{3}H]neurotensin binding was measured on homogenates and intact neurons cultured for 5 days. [^{3}H]Neurotensin (2 nM) was incubated with the homogenate at 25° for 20 min

before the addition of 1 μM of unlabeled neurotensin. As shown in Fig. 4A, there was a rapid and almost complete dissociation of the labeled ligand from its receptors in neuronal cell homogenates. On the contrary, when intact neurons were incubated for 1 hr at 37° in the presence of [^{3}H]neurotensin (2 nM), rinsed twice with PBS and homogenized, addition of 1 μM unlabeled neurotensin to the homogenate prepared from these cells induced only a slight dissociation of the ligand from its binding sites (Fig. 4B).

The addition of 1 μM of unlabeled neurotensin to intact neurons incubated previously for 1 hr at 37° with [^{3}H]neurotensin induced only a 50% dissociation (or release) of the ligand (Fig. 4C).

Figure 4D shows the dissociation of the ligand from intact neurons incubated with [^{3}H]neurotensin for 1 hr at 0–4°. The dissociation, measured at 37° in the presence of a large excess of unlabeled peptide, was rapid and almost complete.

[^{3}H]*Neurotensin receptor regulation*

The regulation of neurotensin receptor number was studied by incubating intact neurons with neurotensin itself and some of its analogs.

Four-day-old intact neurons were incubated with 3 nM unlabeled neurotensin (1–13), (1–8) or (8–13) at 0–4° or 37° for 1 hr.

The incubation medium was then removed, the cells were rinsed three times with PBS solution and the [^{3}H]neurotensin binding (2 nM) was measured on homogenates prepared from these neurons (Table 3).

When intact neurons were incubated for 1 hr with neurotensin (1–13) at 37°, [^{3}H]neurotensin binding was reduced to 37% of control values. Saturation binding experiments were performed on control cell homogenates and on homogenates of neurons preincubated with unlabeled neurotensin. Scatchard analysis of the saturation curves revealed that the reduced binding observed on treated cells corresponded to a decrease of the B_{max} value of about 60% without any significant modification of the equilibrium dissociation constant (K_D value) (Fig. 5). When intact neurons were incubated for 1 hr at 37° with 3 nM of the active neurotensin fragment (8–13), the decrease of the specific binding was in the same range as that observed when neurotensin (1–13) was used.

Incubation of intact neurons at 0–4° with neurotensin (1–13) (3 nM) or at 37° with the inactive fragment (1–8) did not induce any significant decrease of the [^{3}H]neurotensin binding (Table 3).

The time course of the neurotensin-induced decrease of [^{3}H]neurotensin specific binding is shown in Fig. 6. Intact cells were incubated for different periods of time in the presence of neurotensin (1–13) at 37°. Cells were rinsed and the [^{3}H] neurotensin binding was determined both on intact neurons (Fig. 6A) and homogenates from the same cells (Fig. 6B). In the typical experiments illustrated, the decrease of the binding reached a maximum after about 30 min in both cases.

The effects of some putative inhibitory drugs on the short-term neurotensin-induced decrease of the [^{3}H]neurotensin binding were examined (Table 4). Pretreatment with bacitracin (3 mM), before further incubation of the intact cells with neurotensin, resulted in a moderate (about 30%) but significant inhibition of the peptide-induced decrease of receptor number measured either on intact neurons or their homogenates. Chloroquine and methylamine, two lysosomotropic drugs, and colchicine and nocodazole, two antimicrotubular agents [31, 32], did not prevent the rapid neurotensin-induced reduction in receptor number.

DISCUSSION

The foregoing results provide evidence for the occurrence of neurotensin receptors in cultured neuronal cells and their regulation induced by the ligand itself.

Two distinct binding sites for neurotensin were described in rat brain and called NT_1 and NT_2 sites. The NT_1 sites were displaced selectively by levocabastine (a known histamine H1 antagonist) whereas the NT_2 sites were referred to as physiological receptors [14]. The NT_1 acceptor sites were not detected in rat brain before day 10 of postnatal life [16]. Since there was no displacement of [^{3}H]-neurotensin binding by 1 μM levocabastine either in cultured neuronal cells from the forebrain of rat embryos or in the cell homogenates, [^{3}H]-neurotensin binding sites correspond to high affinity neurotensin receptors. Neurotensin (1–13) and the active fragment (8–13) of neurotensin inhibited the binding of [^{3}H]neurotensin with IC_{50} values that are in agreement with those obtained in adult rat cortex (14). In contrast, the fragment (1–8) of neurotensin was inactive.

The number of receptors in neuronal cells differed greatly according to the age of the culture: a marked increase in the B_{max} values (but not in the K_D values) of the neurotensin receptors was observed in homogenates of 10-day-old as compared to those of 5-day-old cultured cells.

This increase of the B_{max} values could reflect a higher expression rate of neurotensin receptors, comparable to that observed in the ontogenic development of the rat brain during the first days after birth. Neurotensin receptors are present in rat brain as early as gestational day 14 [33]. The receptor number increases until postnatal day 10–15 and declines afterwards [16, 33–35].

When [^{3}H]neurotensin binding was measured at 0–4° in intact neurons, the mean B_{max} value was not significantly different from that measured at 37° (Table 1). A similar high affinity was observed either when the binding was performed in intact neurons at 0–4° or 37°, or with a total particulate fraction prepared from the same cells.

These results of binding experiments performed with cultured neurons suggest that most neurotensin receptors measured in intact neurons are localized at the cell surface.

The association of [^{3}H]neurotensin to receptor sites in intact neuronal cells was rapid at 0–4° and at 37° (Figs 2 and 3). When the association experiment was performed at 0–4° in isotonic buffer, or in culture medium containing bacitracin (0.2 mM), the binding remained stable for at least 4 hr. This

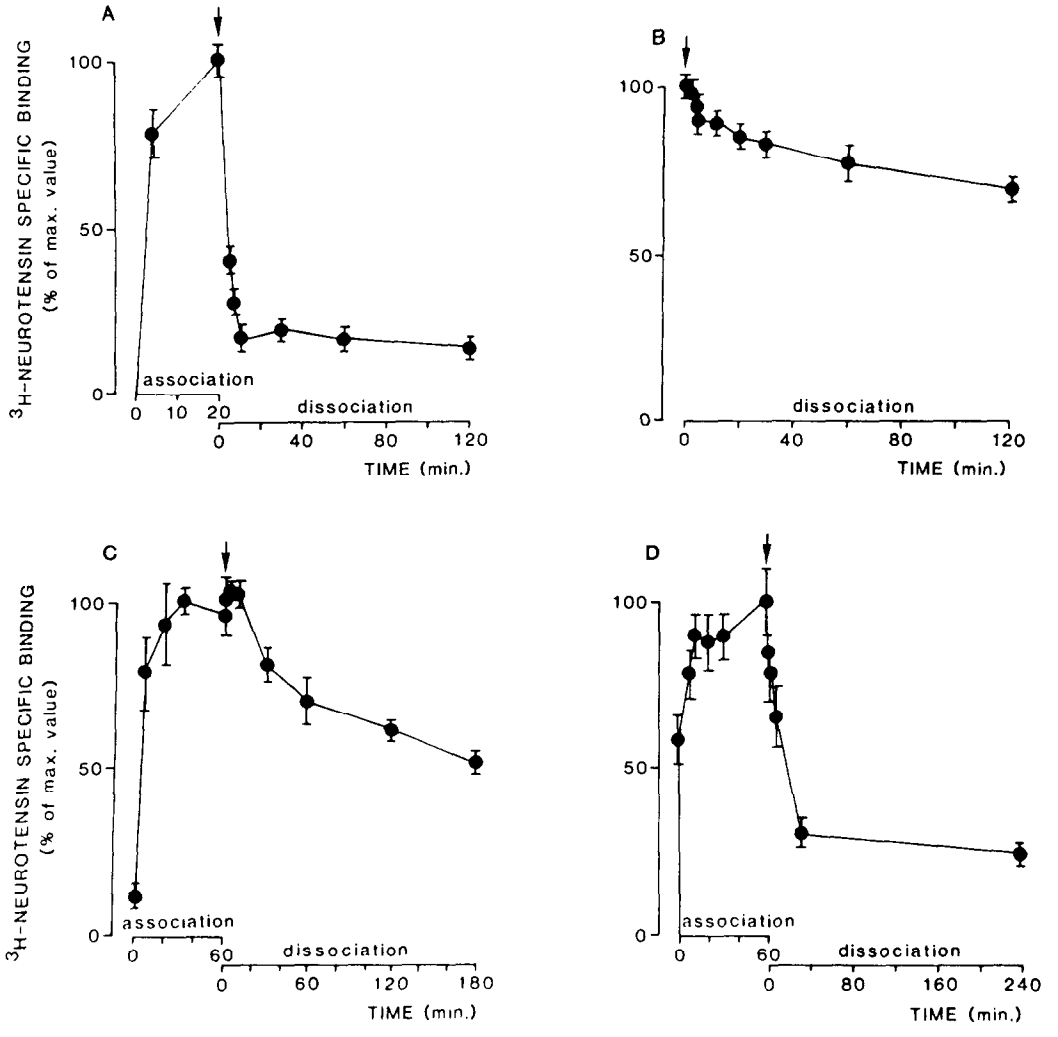

Fig. 4. (A) Kinetics of dissociation of [^{3}H]neurotensin binding from cell membrane homogenate. Homogenates from cultured neurons were incubated at 25° for 20 min with [^{3}H]neurotensin (2 nM). Unlabeled neurotensin (1–13) (1 μM) was added to the incubation medium and the binding was measured after different times. Results are expressed as means ± SEM of three different experiments. (B) Kinetics of dissociation of [^{3}H]neurotensin binding in homogenate obtained from intact cultured neurons that were incubated with [^{3}H]neurotensin at 37°. The specific binding of [^{3}H]neurotensin (2 nM) was determined in intact cultured neurons incubated at 37° for 1 hr. Cells were rinsed twice, homogenized and suspended in binding buffer at 37°. Unlabeled neurotensin (1 μM) was added to the homogenates and the [^{3}H]neurotensin binding was measured after different periods of time. Results are expressed as means ± SEM of three different experiments. (C) Kinetics of dissociation of [^{3}H]-neurotensin binding measured at 37° on intact neurons. Intact neurons were incubated at 37° for 1 hr with [^{3}H]neurotensin (2 nM) and the specific binding was determined. Then, 1 μM neurotensin was added to the incubation medium and the binding was measured at different times after further incubation of the intact cells at 37°. Results are expressed as means ± SEM of three different experiments. (D) Kinetics of dissociation of [^{3}H]neurotensin binding measured at 37° on intact neurons previously incubated with the ligand at 0–4°. Intact neurons were incubated at 0–4° for 1 hr with [^{3}H]neurotensin (2 nM) and the specific binding was determined. Then, 1 μM unlabeled neurotensin was added to the incubation medium and the binding measured at different times after further incubation of the intact cells at 37°. Results are expressed as means ± SEM of three different experiments.

Table 3. [^{3}H]Neurotensin binding (% of control values) in 4-day cultured cells treated with neurotensin (1–13) or (1–8), 3 nM, at 0–4 or 37°

	0–4°	37°
Control	100 ± 26 (5) (100.4 ± 26.0 fmol/mg protein)	100 ± 10 (27) (81.6 ± 8.6 fmol/mg protein)
Cells incubated with neurotensin (1–13) for 1 hr	105 ± 7 (4)	37 ± 4 (24)
Cells incubated with neurotensin (1–8) for 1 hr	—	100, 81 (2)

Mean values ± SEM of [^{3}H]neurotensin specific binding in (N) different experiments performed in triplicate are indicated and the control values are also expressed in fmol/mg protein.

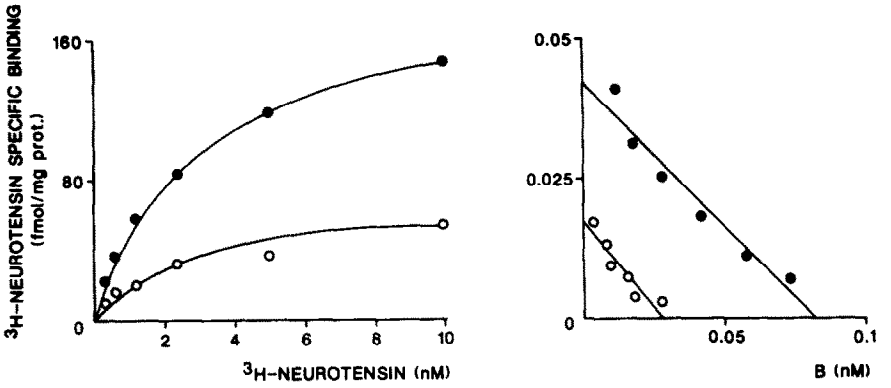

Fig. 5. Saturation curves and Scatchard analysis of [^{3}H]neurotensin specific binding to homogenates from neuronal cells. Control conditions (culture of 4 days), (●); cells incubated for 1 hr at 37° with neurotensin (1–13) (3 nM). (○) Inset: Scatchard analysis of the specific binding. The K_D and B_{max} values were, respectively, 1.95 nM and 165.3 fmol/mg protein in control conditions and 1.66 nM and 56.6 fmol/mg protein in neurotensin-treated cells. B, concentration of [^{3}H]neurotensin specifically bound (nM).

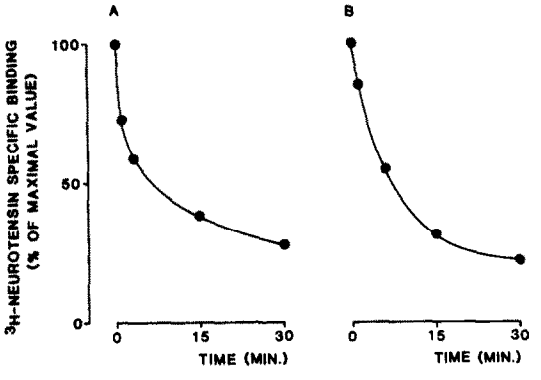

Fig. 6. Time course of neurotensin-induced decrease of [^{3}H]neurotensin specific binding determined in intact neurons (A) and homogenates (B) from these cells. Intact neurons were incubated with 3 nM neurotensin (1–13) at 37° for various periods of time. At the end of the incubation, [^{3}H]neurotensin binding was measured on intact cells at 0–4° or in a total particulate fraction from the same cells at 25° with a single concentration of [^{3}H]neurotensin (2 nM).

indicates that the degradation of the peptide was reduced under these experimental conditions and that at 37° the activity of the peptidases or other proteases secreted in the medium, or located at the external side of the cell membrane, was in part responsible for the progressive degradation of the peptide. This degradation has been described previously in mouse cultured neurons [25].

Time course of [^{3}H]neurotensin association revealed a progressive decrease of the labeling when intact cultured neurons were maintained at 37° for more than 1 hr (Fig. 3). This decrease was observed both in the presence and in the absence of 1,10-phenantroline (1 mM) and bacitracin (0.2 mM). This could correspond to a progressive intracellular degradation of the peptide despite the peptidase inhibitory effect of bacitracin. In the same way, a decline of ^{125}I-labeled epidermal growth factor binding to human fibroblasts was observed when the intact cells were incubated at 37° for more than 1 hr. This was not due to degradation of the free [^{125}I]-labeled epidermal growth factor in the incubation medium but was thought to correspond to degradation of the ligand–receptor complex consecutive to its internalization [36].

Several lines of evidence suggest that neurotensin receptor number is regulated by agonists through an internalization process.

Table 4. Effect of drugs on short-term (1 hr) neurotensin-induced loss of [^{3}H]neurotensin specific binding

Incubation with drugs or peptides	[^{3}H]Neurotensin specific binding, % of control value (absolute value)
None (control)	100 ± 10 (N = 19) (61.9 ± 5.8 fmol/mg protein)
Neurotensin (1–13) 3 nM	40 ± 4 (N = 19) (24.8 ± 2.8 fmol/mg protein)
Neurotensin (1–13) 3 nM	
+bacitracin 3 mM (a)	65 ± 8 (N = 5)*
+colchicine 10 μM (b)	51 ± 11 (N = 5) NS
+nocodazole 10 μM (c)	31 ± 9 (N = 3) NS
+methylamine 10 mM (a)	38 ± 13 (N = 4) NS
+chloroquine 100 μM (a)	40 ± 8 (N=3) NS

Intact cells were preincubated for 30 min (a), 4 hr (b) or 2 hr (c) before further incubation with the same compounds in the presence of unlabeled neurotensin (3 nM) for 1 hr at 37°.

[^{3}H]Neurotensin specific binding was measured on the total particulate fraction from treated cells and results are expressed as percent value of the control experiments (cells not treated with neurotensin) that were always performed at the same time and under the same conditions.

NS, not significant; * $P < 0.05$, Student's t test.

First, when intact neurons were incubated in the presence of 3 nM unlabeled neurotensin (1–13), the specific [^{3}H]neurotensin binding was decreased. The incubation of intact cells for 15 min at 37° with the peptide was sufficient to reduce the binding capacity to less than 50% of the controls (Fig. 6). The association curve of [^{3}H]neurotensin in intact cultured neurons displays kinetics similar to those of the neurotensin-induced decrease of receptor number (Figs 2 and 3).

Since in intact cells, [^{3}H]neurotensin only binds to the cell surface receptors either at 0–4° or 37°, the receptor disappearance from the cell surface has to occur immediately after neurotensin binding. Interestingly, incubation of intact neurons with neurotensin at 0–4° did not lead to reduction of the [^{3}H]neurotensin binding (Table 3).

When a cell homogenate from cultured neurons was incubated at 37° with unlabeled peptide and rinsed twice before receptor determination, there was no decrease in [^{3}H]neurotensin binding capacity. This observation and the lack of change in binding affinity in neurotensin-treated cells indicate that the neurotensin-induced decrease of receptor number in cultured neurons was not due to receptor occupancy by neurotensin remaining after incubation and washing.

The rapid disappearance of cell-surface receptor ([^{3}H]neurotensin specific binding measured at 0–4° on intact neurons) triggered by neurotensin during incubation of the neurons at 37°, corresponded to a decrease in receptor number (decrease of the B_{max} value).

Therefore, ligand–receptor binding, cell integrity and incubation at physiological temperature are needed to reveal receptor internalization.

Second, the neurotensin-induced decrease of receptor number was partially inhibited by bacitracin which, besides its inhibitory effects on peptidase activity, is known to inhibit the clustering-endocytotic process [31, 37]. The effect of this compound suggests that the first step in neurotensin-induced receptor regulation is the mobilization of these receptors inside the cell membrane.

Third, the neurotensin-induced decrease of neurotensin receptors was observed both at the cell surface of intact neurons and in the homogenates of the treated cells (Fig. 6). This could be explained either by an intracellular receptor sequestration or by receptor degradation after their internalization (true down-regulation).

The neurotensin-induced decrease of neurotensin receptors in homogenates suggests that once the receptors have been internalized [^{3}H]neurotensin is no longer able to reach them. According to this hypothesis, we observed that after 1 hr of incubation of intact neurons with [^{3}H]neurotensin at 37° the binding was apparently irreversible (Fib. 4B and C). Such "irreversibility" is compatible with the sequestration of [^{3}H]neurotensin in membrane compartments or vesicles from which it can not be released. Receptor internalization might be followed by sequestration within vesicles which could thereafter slowly migrate towards the cell body, the nucleus or the lysosomes. Such intracellular retrograde transport of receptor-bound neurotensin was shown to occur from the striatum to the substantia nigra in rat brain [32].

Receptor degradation seems unlikely to explain fast receptor disappearance because such a degradation process implies internalization, transport and lysosomal delivery. Moreover, antimicrotubular agents (colchicine and nocodazole) and lysosomotropic drugs (chloroquine and methylamine) were without effect on the reduction of receptor number induced within 1 hr by neurotensin.

Therefore, the rapid decrease in receptor number does not correspond to lysosomal degradation. The intracellular transport along microtubules (at least those sensitive to colchicine or nocodazole) does not seem to be involved.

Finally, we present evidence for a rapid decrease of cell surface receptors which is parallel to [^{3}H]-neurotensin uptake in intact neurons. This is compatible with the receptor-mediated endocytosis of neurotensin.

Neurotensin, along with many other transmitters and neuropeptides, binds to a specific receptor leading to second messenger activation and physiological response. Moreover, the binding of neurotensin to its receptors also leads to a process of receptor internalization which appears to be the first step in receptor regulation and which allows through the same process the specific endocytosis of the peptide.

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