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INTERNALIZATION AND INTRACELLULAR MOBILIZATION OF NEUROTENSIN IN NEURONAL CELLS

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The binding of peptide ligands to cell surface receptors is commonly followed by internalization of receptor–ligand complexes (for a review see Refs. 1–3). In the nervous system, this biological process has mainly been documented for large polypeptide molecules such as growth factors [4, 5]. However, a growing body of evidence supports the view that certain neurotransmitters may also be internalized as a result of their interaction with neural receptors. Thus, internalization of muscarinic [6–8], β-adrenergic [9, 10], GABA-benzodiazepine [11] and neurotensin [12, 13] receptors has been documented in neuronal and/or glial cell cultures and in brain slices *in vitro*. Furthermore, there is evidence that ligands internalized at the level of axon terminals (i.e. presynaptically) may be retrogradely transported towards the cell body *in vivo*. Thus, [^{3}H]lofentanyl, a selective μ opioid agonist, was shown to be retrogradely transported in the rat vagus nerve [14, 15], and ^{125}I-neurotensin to be retrogradely transported in the nigro-striatal pathway following its intracerebral injection into the rat neostriatum [16, 17]. That this retrograde transport involves internalized receptor–ligand complexes and not merely the radioactive ligand itself is supported by the demonstration of downstream opioid and neurotensin receptor pile-up subsequent to the ligation of opioid/neurotensin-receptive pathways [18, 19]. In contrast, other neuropeptide receptors, such as those for cholecystokinin, do no accumulate downstream from the ligation [20], suggesting that either not all neuropeptide receptors are internalized or that the internalization process is not systematically followed by axonal transport of receptor–ligand complexes.

The key to our understanding of the role of neurotransmitter internalization and internalization-induced axonal transport rests in part on the elucidation of the cellular mechanisms and cytological substrates subserving these biological processes. Indeed, whereas studies carried out on non-neuronal cells have identified the endosomal compartment as a major player for receptor-mediated endocytosis and subsequent intracellular migration of internalized molecules [21, 22], little is known of the intracellular compartments subserving this function in neurons. In fact, studies of neuronal endocytosis have mainly focused on the retrieval of membrane after neurotransmitter release by nerve terminals [23], and have only recently addressed the question of the sorting of classical endocytic markers such as horseradish peroxidase or transferrin [24]. There is virtually no information available on the sequence of events leading to the internalization of neurotransmitters into their target cells. The present paper offers preliminary insight into the mechanisms subserving transmitter internalization through a review of complementary biochemical, autoradiographic and confocal microscopic studies of the internalization of the neuropeptide, neurotensin (NT), in neuronal cell cultures *in vitro* and in the central nervous system *in vivo*.

Studies in neuronal cell cultures

The experiments to be described were carried out using either monoiodo neurotensin (^{125}I-NT) or *Nα* fluoresceinyl-thiocarbamyl(FTC)-[Glu^1]neurotensin (FTC-NT; fluo-NT). The iodinated ligand was prepared by a lactoperoxidase H_2O_2 method and purified (sp. act. 2000 Ci/mmol) on an ion-exchange column as described [25]. The fluorescent ligand was synthesized using a solid phase methodology based on t-Boc chemistry/acid-labile amino acid protection and purified at 99% homogeneity by HPLC [26]. Both of these ligands were shown to bind to mouse brain membrane preparations with the same affinity and selectivity as native NT [25, 26] and to label selectively high affinity NT receptors in rat brain sections *in vitro* [26, 27].

Biochemical experiments. The binding and internalization of ^{125}I-NT was first investigated biochemically in intact neuronal cells in culture. Two types of preparations were utilized: primary cultures of neurons derived from whole embryonic mouse brain and neuron–neuroblastoma hybrid cells (SN-17) produced by fusion of embryonic mouse septal cells with a murine neuroblastoma. The SN17 cell line was kindly provided to us by D. N. Hammond [28] and N. R. Cashman. The choice of these two model systems was based on the demonstration that both exhibited high levels of specific high affinity NT binding sites [29, 30]. SN-17 cells offered the

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additional advantages of being immortalized and phenotypically cholinergic [31].

In a first set of experiments designed to assess the effects of time and temperature on the kinetics of association of ^{125}I-NT, neurons and SN17 cells were incubated for 5–60 min at 4° or 37° with 0.2 nM ^{125}I-NT in Earle's HEPES buffer, in the presence of peptidase inhibitors, and with or without a 100-fold excess of native NT for determination of non-specific binding. In each type of cells, specific ^{125}I-NT binding was saturable at both 4° and 37°, and plateaued after 40–60 min of incubation [32, 33]. However, the total amount of peptide specifically bound at equilibrium was substantially higher at 37° than at 4° in both preparations. Furthermore, whereas the maximal binding capacity (B_{max}) at 4° was comparable to that observed on membrane homogenates prepared from the same type of cells, the one observed at 37° was consistently higher, suggesting that the B_{max} at 4° corresponded to the original number of cell surface receptors whereas the one at 37° reflected a temperature-dependent increase in the number of specific ^{125}I-NT binding sites [32, 34]. Two different lines of evidence were to indicate that this increase in the number of binding sites was a consequence of receptor internalization. First, when ^{125}I-NT was incubated with neurons at 37° but in the presence of 10 μM of the endocytosis inhibitor, phenylarsine oxide, the maximal specific binding was similar to that seen at 4°, (i.e. 160 fM/mg protein; [32, 34]). Second, when cells incubated at 37° were subjected to high sodium (0.5 M NaCl), basic (pH 9) or acid (pH 2.5) washes to extract surface-bound radioactivity, a significant fraction of the tracer was recovered in the non-washable (i.e. intracellular) fraction (Fig. 1). As can be judged from the evolution of this fraction with time (Fig. 1), the internalization process is rapid and saturable. The calculated $T_{1/2}$ of internalization was of approximately 10 min for neurons and 13 min for SN17 cells [32, 33]. As can be seen in Fig. 2, the capacity of the internalization process in neurons is a function of the concentration of the peptide and reaches its half-maximal effect at a concentration of 0.3 nM [34]. This value corresponds to the K_D of ^{125}I-NT binding to neurons in the presence of phenylarsine oxide, suggesting that the internalization process is itself receptor-dependent.

Autoradiographic experiments. In a second set of experiments, the distribution of bound ^{125}I-NT was examined by light microscopic autoradiography in cultured neurons labeled as above with 0.2 nM ^{125}I-NT at either 37° or 10° [13]. In these experiments, 10° was preferable to 4° as it allowed better morphological preservation without significantly increasing ligand internalization, at least within the time frame of our experiments. At all time intervals examined (5, 30 and 60 min), specifically bound radioactivity was confined to selective neuronal subsets (Fig. 3a). As illustrated in Fig. 4, the density of ^{125}I-NT labeling increased with both the duration and the temperature of incubation. However, whereas the density of silver grains detected over nerve cell bodies and processes remained proportionally the same between 5 and 30 min when the incubations were carried out at 10°, the proportion of grains associated with neuronal perikarya (as opposed to neuronal processes) was much greater at 37° than at 10° (Fig. 4).

Autoradiograms of 1 μm-thick plastic-embedded sections taken through ^{125}I-NT-labeled cells revealed a prominent intracellular localization of the label in neurons incubated at 37° (Fig. 3b). This intracellular labeling was already apparent after 5 min of incubation but was most prominent after 30 and 60 min of labeling (Fig. 3b), in keeping with our biochemical data. By contrast, there was virtually no intracellular labeling in cells labeled at 10° at all times examined.

These results provide definitive evidence for the occurrence of a time- and temperature-dependent internalization of ^{125}I-NT in neuronal cells *in vitro*. Studies in semi-thin sections clearly indicate that the difference in cell labeling density between 10° and 37° is mainly attributable to an increase in intracellular radioactivity. It can therefore be surmised that the proportionally higher labeling of neuronal perikarya as compared to processes observed in whole cell preparations (Fig. 4) reflects either a greater internalization of the label at the level of the perikarya or, more likely, mobilization of the internalized ligand from the processes towards the cell bodies.

Confocal microscopic experiments. Confocal microscopic analysis of the internalization process was carried out on both SN17 cells and primary neurons in culture, using fluo-NT as a marker. Neurons used for these experiments were derived from the mesencephalon of embryonic rat brain in which high affinity NT binding sites had been shown to be preferentially associated with cells immunoreactive for tyrosine hydroxylase [35].

The effect of temperature on the cellular distribution of bound fluo-NT was examined in SN17 cells. Briefly, the cells were incubated for 60 min at 4° with 20 nM fluo-NT in Earle's HEPES buffer containing 10 μM 1,10 phenanthroline. At the end of the incubation, the cells were either immediately washed and air dried for confocal microscopic viewing, or warmed up at 37° for a further 45 min before confocal microscopic examination.

Serial optical sectioning of cells incubated at 4° revealed the label to be confined to the cell surface (Fig. 5a). The absence of intracellular staining was particularly striking in thin (0.12 μm-thick) optical sections passing through the plane of the nucleus. On the surface of the cell, the marker formed small irregular hot spots, 0.5–1 μm in diameter (Fig. 5a). In cells that were warmed up for a further 45 min at 37° after labeling of cell surface receptors, the signal had all but disappeared from the surface of the cells, while the cytoplasm had become filled with small, rounded, intensely fluorescent granules [29].

The effect of time on the intracellular distribution of internalized fluo-NT was examined in embryonic mesencephalic neurons. Briefly, the cells were incubated at 37° in Earle's HEPES buffer containing 20 μM fluo-NT in the presence of 10 μM 1,10 phenanthroline and examined 5, 15, 30 and 60 min later under the confocal microscope. After 5 min of incubation, the label appeared highly granular and was mainly concentrated at the periphery if not at

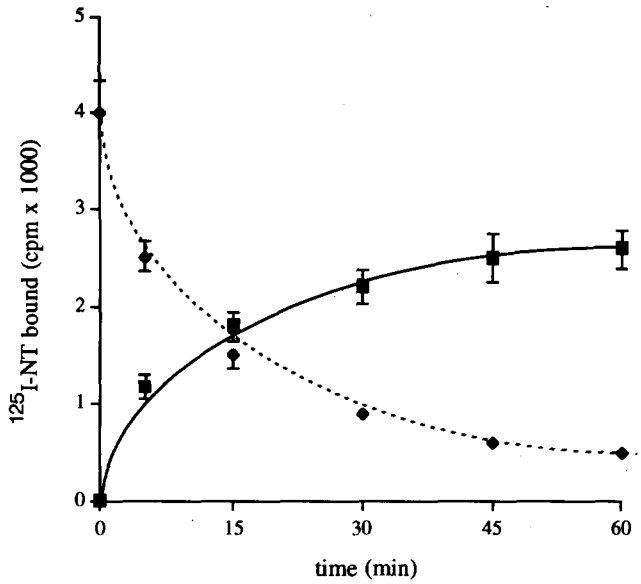

Fig. 1. Comparative evolution of acid-resistant (■) and acid-washable (●) fractions of ^{125}I-NT specifically bound to intact SN17 cells as a function of time. Cells were incubated with 0.2 nM ^{125}I-NT at 4° for 1 hr to ensure saturation of cell surface receptors, after which they were transferred to 37° for 5–60 min. At the end of the incubation, cells were washed in hypertonic acid (pH 2.5) buffer to separate surface-bound (acid-washable) from internal (acid-resistant) radioactivity. Values are means of three different experiments carried out in duplicate.

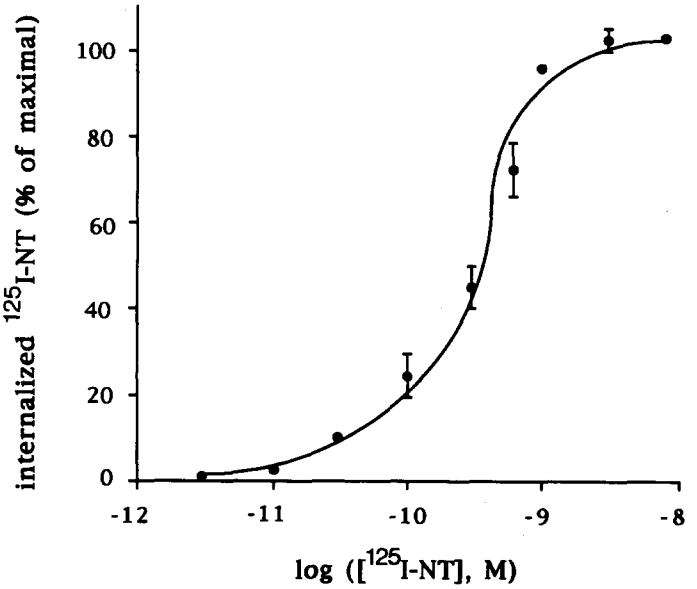

Fig. 2. Dependence of ^{125}I-NT internalization on the concentration of extracellular NT. Cells were incubated with increasing concentrations of ^{125}I-NT for 45 min at 37° and acid-washed at the end of the incubation to differentiate internalized (acid-resistant) from cell surface (acid-washable) radioactivity. Proportion of internalized ^{125}I-NT expressed as a percentage of maximal value of internalized fraction. Values are means ± SEM from three independent experiments carried out in duplicate. Reproduced from *Methods in Neuroscience Vol. 11, Receptors, Model Systems and Specific Receptors*, pp. 334–351, 1993 [34] with permission.

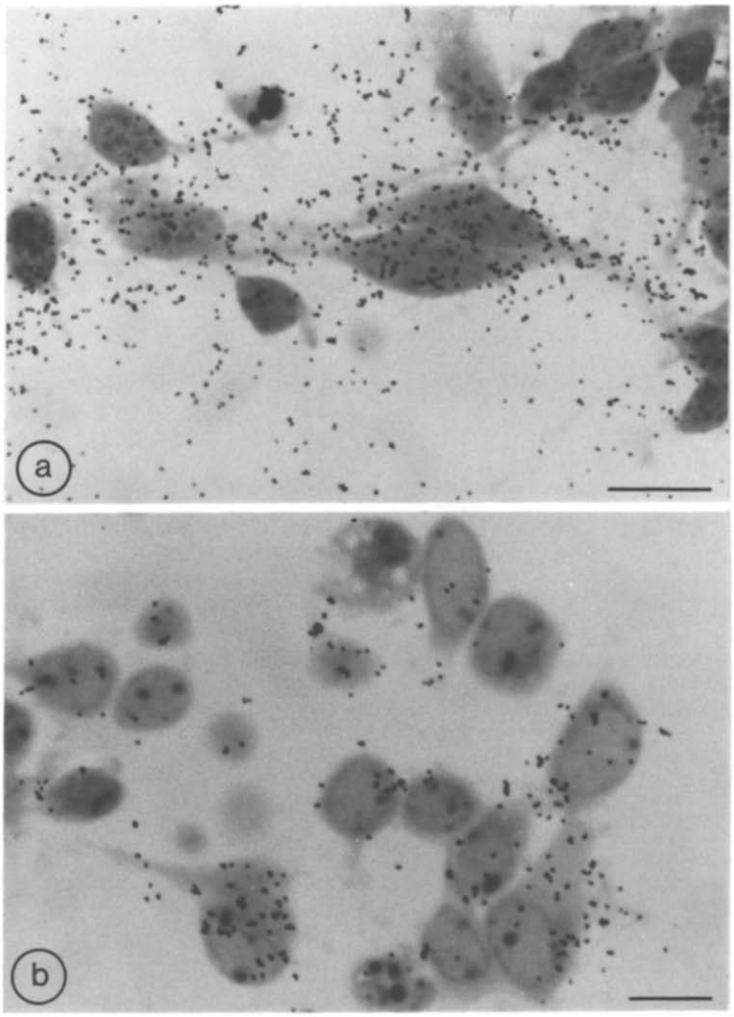

Fig. 3. Light microscopic autoradiograms of neurons labeled in culture after incubation with 0.2 nM ^{125}I-NT at 37°. (**a**) Cells processed whole after 5 min of exposure to the radioligand. Note the selective accumulation of silver grains over three fusiform neuronal perikarya and their processes. (**b**) Autoradiogram of a 1-μm thick plastic section taken across cells incubated with the ^{125}I-NT for 60 min. Three of the cross-sectioned neurons show pervasive labeling of their cytoplasm but comparative sparing of their nuclei. Toluidine blue stain. Scale bars: 10 μm. Reproduced from *Brain Res* **564**: 249–255, 1991 [13] with permission.

the surface of the cells' perikarya and processes (Fig. 5b). Immunohistochemical counterstaining of the cultures with an antibody against tyrosine hydroxylase confirmed that the vast majority of the fluo-NT-labeled cells were catecholaminergic in nature (Fig. 5b). After 15 min of incubation, the ligand pervaded the cytoplasm of cell bodies and processes alike in the form of small, rounded, intensely fluorescent particles (Fig. 5d, e). By 30 min, the intracytoplasmic labeling was still granular but was proportionally more intense at the level of perikarya than at that of neuronal processes (Fig. 5c). Finally, at 1 hr, the labeling showed little increase in intensity but appeared somewhat more concentrated in the perinuclear region than at the periphery of the cell.

These confocal microscopic observations provide additional evidence for a temperature- and time-dependent internalization of NT subsequent to its specific binding to neuronal cells *in vitro*. Furthermore, they demonstrate that the internalized peptide is sequestered within small, endosome-like particles which are mobilized with time from the processes towards the perikaryon and, specifically, the perinuclear region.

Retrograde axonal transport of internalized neurotensin in nigro-striatal neurons in vivo

As mentioned previously, *in vivo* administration of ^{125}I-NT in the neostriatum of adult rats in the presence of the endopeptidase 24.11 inhibitor kelatorphan was shown to result in selective retrograde labeling of neurons in the substantia

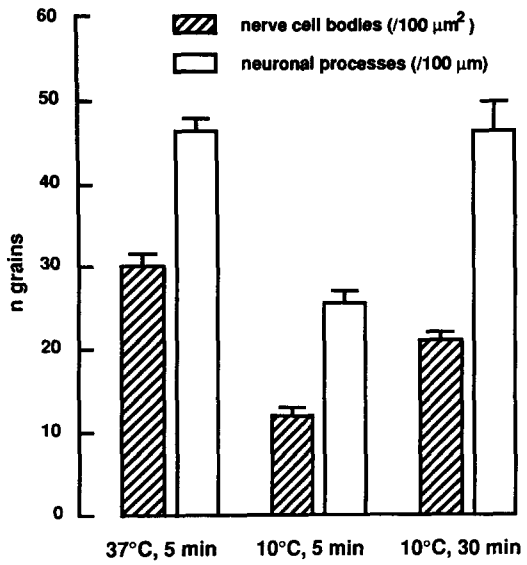

Fig. 4. Quantitative analysis of silver grain distribution in ^{125}I-NT-labeled mouse embryonic neurons. Cells were autoradiographed whole after 5 min of incubation at 37° or 10°, or after 30 min of incubation at 10°. Labeling of processes expressed as number of grains per unit length. Reproduced from *Brain Res* **564**: 249–255, 1991 [13] with permission.

nigra, pars compacta [16]. This retrograde transport was reportedly microtubule-dependent, in that it was abolished by intracerebral administration of colchicine, and receptor-induced, in that it was selectively prevented by co-injection of non-radioactive NT_{1-13} or NT_{8-13}, but not of the inactive fragment NT_{1-8} [16]. Further evidence that this retrograde labeling was receptor-mediated stemmed from its selectivity towards nigral neurons, which have long been known to contribute the bulk of presynaptic NT receptors to the neostriatum [36, 37]. In an attempt to gain insight into the cellular mechanisms responsible for this retrograde labeling, we examined the subcellular distribution of retrogradely transported NT using both confocal and electron microscopy and have compared this distribution to that observed following internalization of the peptide in cell cultures.

Confocal microscopic studies. Adult rats were injected stereotaxically under sodium pentobarbital anesthesia (65 mg/kg i.p.) with 2 μL of kelatorphan (15 mg/mL) followed 10 min later by 0.5 nmol of fluo-NT into the left caudoputamen. The animals were killed 4.5 or 8 hr later by perfusion with 4% paraformaldehyde and the brains cut on a freezing microtome after overnight cryoprotection in 30% sucrose. Sections of the midbrain were examined by confocal microscopy before of after immunohistochemical counterstaining with an antibody against tyrosine hydroxylase.

At both times studied, confocal microscopic examination of the midbrain sections revealed extensive fluorescent labeling of nerve cell bodies and their proximal dendrites in the substantia nigra, pars compacta. As expected from the demonstrated association of presynaptic NT receptors with dopamine axon terminals in the neostriatum [36, 37], virtually all of the retrogradely labeled cells were found to be tyrosine hydroxylase-immunopositive in our dual labeling experiments. Both 4.5 and 8 hr after intracerebral administration of fluo-NT, the labeling pattern in nigral neurons was intensely granular (Fig. 5f–h). However, serial optical sectioning at 0.5 μm thickness revealed marked differences in the size and distribution of fluorescent granules between each time point. At 4.5 hr, fluorescent particles were small (2.5 μm in mean diameter), numerous and distributed throughout the cytoplasm of the cells (Fig. 5f). By contrast, after 8 hr, intracellular fluorescent particles were larger (4.3 μm in mean diameter), less numerous and clustered against the nuclear membrane (Fig. 5g, h). These images were reminiscent in many respects of those of neuronal cells in culture in which the internalized ligand was similarly observed to be sequestered in particulate, presumably vesicular form and to migrate with time from the periphery to the center of the cells. They further suggested that once they have reached the perikaryon, these sequestration compartments coalesce in the perinuclear zone.

Electron microscopic studies. To elucidate further the subcellular compartmentation of the retrogradely transported ligand, electron microscopic autoradiography was carried out on sections of the midbrain 4.5 hr after injection of ^{125}I-NT into the neostriatum [38]. In these preparations, retrogradely transported radioactivity was detected in the form of isolated silver grains scattered over the cytoplasm of nigral perikarya and dendrites [17, 38]. Quantitative analysis of silver grain distribution revealed a predominant association of the retrogradely transported label with the cytoplasm of the cells and/or intracytoplasmic organelles (77% of somatic grains; Fig. 6a–c). The remainder were detected over the nucleus (16%; Fig. 6a, d) and the perikaryal membrane (7%). When the intracytoplasmic distribution of somatic grains was analysed by direct scoring, i.e. by identifying the underlying structure without correcting for possible cross-fire, a significant proportion of the grains were found to overlie rough endoplasmic reticulum and mitochondria (Figs. 6a, 7). Others were clearly associated with lysosomes (Figs. 6c, 7), the Golgi apparatus (Figs. 6a, 7), and multivesicular bodies (Fig. 7). However, a large fraction of the grains could not be directly ascribed to any particular underlying organelle (Fig. 7).

There are both similarities and differences in the distribution of retrogradely transported NT as revealed by high resolution autoradiography and confocal microscopy. On the one hand, the association of silver grains with organelles such as Golgi vesicles, multivesicular bodies and lysosomes conforms to the granular appearance of the fluorescent signal detected by confocal microscopy and is consistent with contemporary models of receptor-induced endocytosis. Indeed, subsequent to internalization, receptor–ligand complexes are currently believed to be sequestered in an acidic endosomal compartment in which they undergo

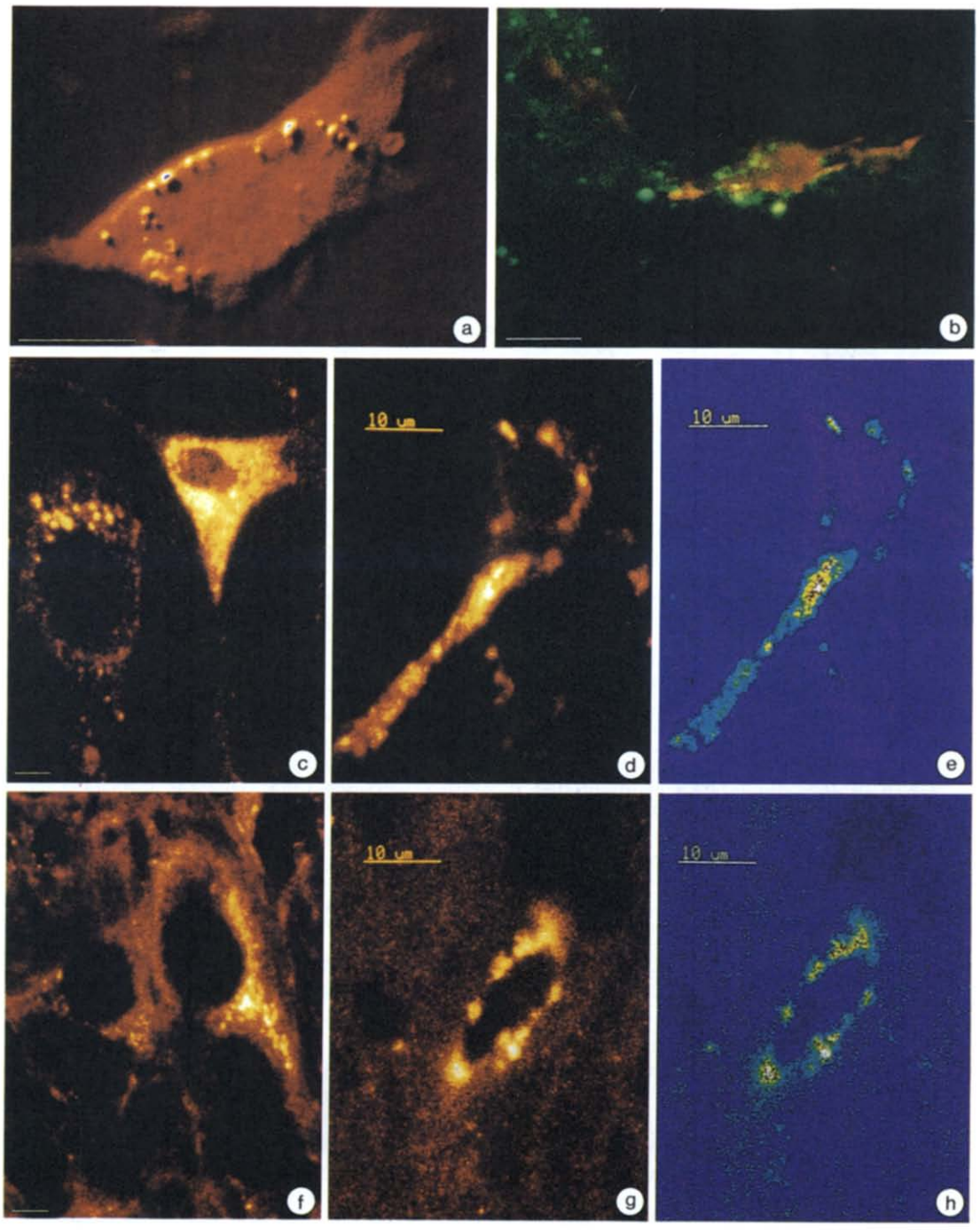

Fig. 5. Confocal microscopic images of neuronal cells labeled *in vitro* (**a–e**) or *in vivo* (**f–h**) with fluo-NT. All images were acquired on a Leica confocal scanning laser microscope (CSLM) consisting of a Diaplan inverted microscope, an argon–krypton laser, a scanning and detection unit and a VME bus computer system. Excitation emission for fluorescein isothiocyanate and Texas red: 488 nm. Scale bars: 10 μm. (**a**) SN17 hybrid cell incubated for 45 min at 4° with 20 nM fluo-NT. Pseudo-three-dimensional image reconstructed from 25 consecutive 0.12-μm thick optical sections. The label is detected in the form of small fluorescent particles, 0.5–1 μm in diameter, which are confined to the cell surface. (**b**) Rat mesencephalic neuron dually labeled with fluo-NT (5 min; 37°) and an antibody against tyrosine hydroxylase revealed with Texas red. Specifically bound fluo-NT molecules are clustered at the periphery of the perikaryon as well as along two neuronal processes. Note that the core of the neuron is devoid of labeling at this time. (**c–e**) Rat mesencephalic neurons incubated with fluo-NT for 15 min (**d, e**) or 30 min (**c**) at 37°. The micrograph in (**e**) is a pseudocolor rendition of the cell illustrated in (**d**) in which zones of highest labeling density are represented in red and zones of low fluorescence intensity represented in blue–purple. At both time intervals, the label is concentrated in small, oval or rounded

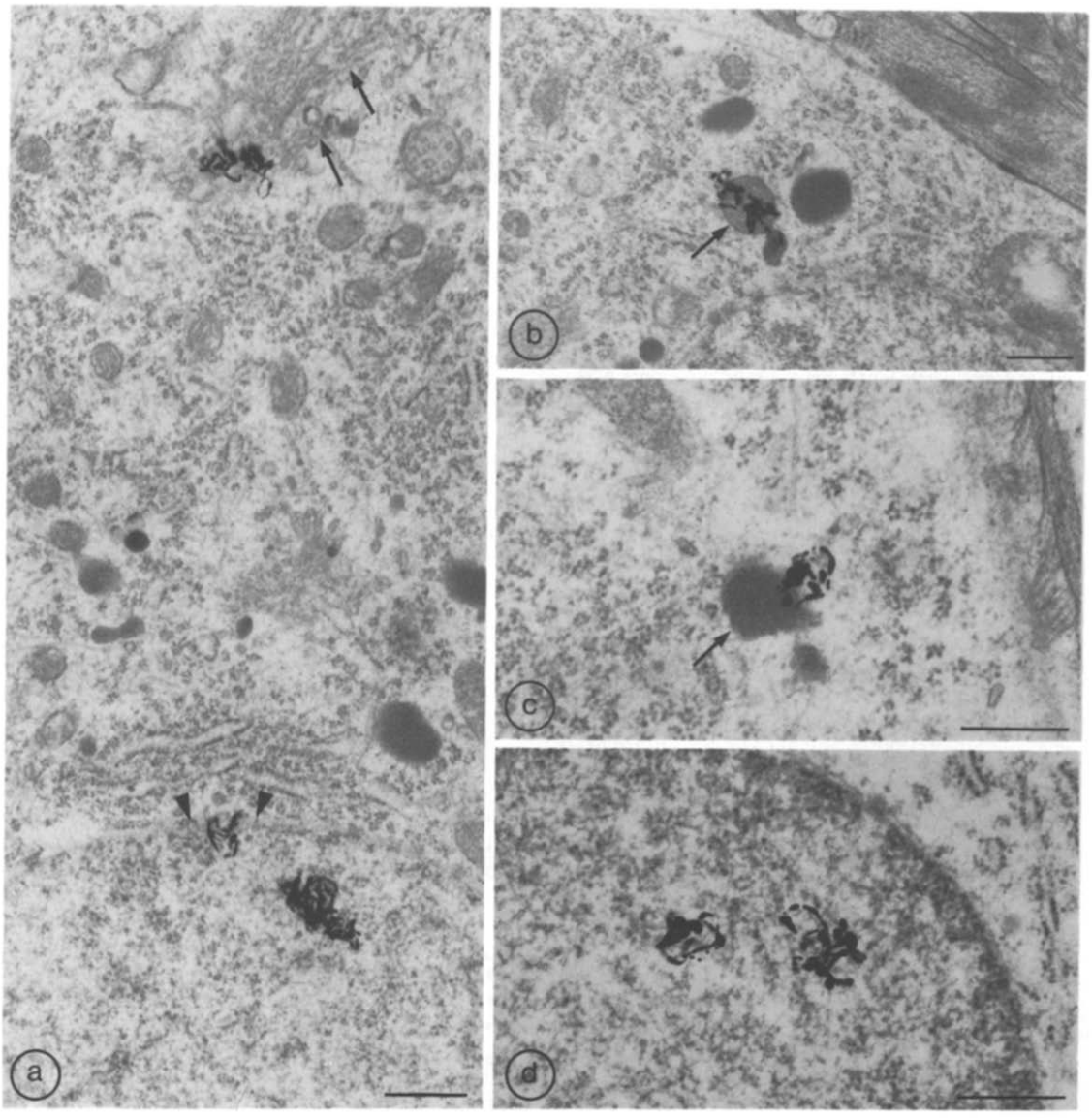

Fig. 6. Electron microscopic autoradiograms of nigral neurons 4.5 hr after ipsilateral intrastriatal injection of ^{125}I-NT. In the cell illustrated in (a), two silver grains are detected over the Golgi apparatus (arrows) and two others over the nucleus (arrowheads). The silver grain in (b) overlies a multivesicular body (arrow) and the one in (c) a lysosome (arrow). The two grains in (d) are detected over a nucleus, next to accumulations of heterochromatin. (a) and (b) reproduced from *Neuroscience* **50**: 269–282, 1992 [38] with permission.

intensely fluorescent particles that pervade the cytoplasm of the cell. However, whereas at 15 min the label is as or more intense in processes than in the perikaryon (**d, e**), at 30 min it is mainly concentrated in the cell body (**c**). Note that in both cases the nucleus remains virtually label free. (**f–h**) Neurons in the rat substantia nigra, pars compacta 4.5 (**f**) and (**g, h**) 8 hr after injection of fluo-NT in the ipsilateral neostriatum. Here again, the image in (**h**) is a pseudocolor transformation of the one in (**g**). Note that the distribution of the label at 4.5 hr is similar to that observed after 30 min of exposure to the ligand in culture. At 8 hr, labeled intracellular granules are markedly larger and clustered against the nuclear membrane (**g, h**). Note the absence of nuclear labeling.

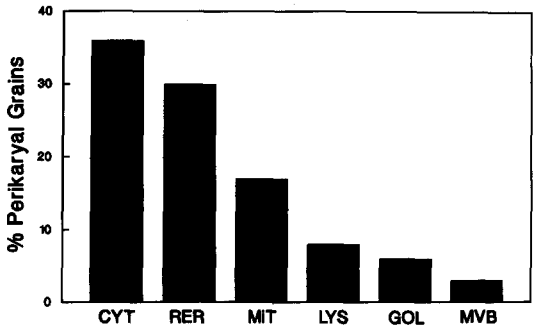

Fig. 7. Distribution histogram of silver grains detected over neurons retrogradely labeled in the substantia nigra 4.5 hr after ipsilateral intrastriatal injection of ^{125}I-NT. Data gathered by direct scoring and expressed as percentage of the total number of perikaryal grains.

dissociation into separate ligand and receptor components [39]. The ligand and receptor are then recycled in the rough endoplasmic reticulum or are translocated to lysosomes for enzymatic degradation. On the other hand, the fact that many of the silver grains showed no obvious association with vesicular organelles is somewhat perplexing and may imply the existence of a non-vesicular pool of internalized ligand. It might also be due in part to the limited resolution of electron microscopic autoradiography which may have led us to ascribe to the cytoplasm silver grains originating from neighbouring vesicular sources. Furthermore, given that only approximately 25–36% of the radioactivity detected in the substantia nigra 4 hr after intrastriatal injection of ^{125}I-NT still corresponds to the unmetabolized peptide [40], it is possible that a major fraction of cytoplasmic grains corresponds to partially hydrolysed fragments of retrogradely transported NT. That these extravesicular molecules would be seen in autoradiographic, but not in confocal images may be due to the fact that electron microscopic autoradiograms were prepared after fixation with glutaraldehyde, which has been shown to cross-link efficiently small peptidic molecules [41], whereas confocal microscopic studies were carried out in tissue fixed with 4% paraformaldehyde, a fixative which would likely have washed away non-sequestered compounds.

The presence of a significant proportion of silver grains over the nucleus was also somewhat unexpected and at odds with our confocal microscopic observations. Here again, the possibility that diffusion of NT degradation fragments from the cytoplasm across the nuclear membrane might be responsible for at least part of the observed nuclear labeling cannot be excluded. However, the nuclear labeling might also reflect an active translocation of NT or of NT receptor–ligand complexes to secondary nuclear targets as extensively discussed elsewhere [17, 38, 42].

Concluding remarks

The studies reviewed here provide compelling evidence for receptor-mediated internalization and subsequent intracellular mobilization of NT in neuronal cells. Both biochemical (acid washes) and anatomical (serial optical or physical sectioning of labeled cells) experiments support the concept of a time- and temperature-dependent entry of the neuropeptide inside nerve cells. That this internalization is receptor-mediated is supported by the fact that its kinetics are similar to those of NT binding to cell surface receptors, and by its selectivity towards neurons documented to harbor high affinity NT receptors (e.g. midbrain dopaminergic cells). The question arises as to whether the process involves internalization of the ligand alone or, as observed in other systems [1–3], of receptor–ligand complexes. Although the studies described herein do not directly address this issue, the fact that receptors have themselves been documented to flow retrogradely within cetain neuronal pathways [18, 19] argues in favor of the latter possibility. Furthermore, preliminary studies carried out in our laboratory on SF9 insect cells transfected with a cDNA coding for the high affinity NT receptor [43] indicate that covalent cross-linking of a nitro-azido derivative of NT to cell surface receptors prior to warming up the cells at 37° does not prevent the entry of the covalently attached probe into the cells as would have been expected had the process involved the ligand alone [44].

The present confocal microscopic studies suggest that once internalized, receptor–ligand complexes are sequestered inside small granular compartments. This observation is in keeping with the results of earlier biochemical studies which have shown that after 1 hr of incubation with [^{3}H]NT at 37°, binding to intact neurons in culture was pseudo-irreversible, i.e. that the addition of unlabeled NT to homogenates prepared from these cells induced only a slight dissociation of the ligand from its binding sites [12]. This pseudo-irreversibility, in turn, presumably accounts for the preservation of the internalized ligand following fixation of cultured cells or brain tissue with paraformaldehyde, whereas the same fixative will not retain NT molecules reversibly bound to cell surface receptors (unpublished observations). The nature of the sequestration compartments is still unclear, but our electron microscopic studies suggest that they correspond in part to endosomal and lysosomal elements.

The present studies also demonstrate an intracellular migration of internalized ligand molecules from distal processes to nerve cell bodies on the one hand, and from the periphery to the perinuclear region of the cell on the other hand. As extensively discussed above, such a pattern of intracellular migration is consistent with current models of receptor degradation and/or recycling following ligand-induced endocytosis. Indeed, current biochemical evidence supports the view that NT internalization induced the incorporation of new receptor units to the membrane in the short term [32] and a down-regulation of cell surface receptors in the long term [12, 45]. By the same token, this mobilization process would ensure removal/inactivation of surface-bound NT. The presence of endopeptidase 24-16, an enzyme shown to play a major role in the functional inactivation of NT

[46, 47], inside NT target cells [48] is consistent with this interpretation. Finally, the perinuclear clustering of internalized ligand molecules, observed here both *in vivo* and *in vitro* in the presence of peptidase inhibitors, suggests further that either the ligand, receptor–ligand complexes or metabolites thereof may be involved, perhaps through a nuclear translocation process, in long-term genomic effects of NT on its target cells [49, 50].

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REFERENCES

1. Bergeron JJM, Cruz J, Kahn MN and Posner BI, Uptake of insulin and other ligands into receptor-rich endocytotic components or target cells: the endosomal apparatus. *Annu Rev Physiol* **47**: 383–403, 1985.
2. Hubbard AL, Endocytosis. *Curr Opin Cell Biol* **1**: 675–683, 1989.
3. Watts C, Endocytosis: what goes in and how? *J Cell Sci* **103**: 1–8, 1992.
4. Bernd P and Greene LA, Electron microscopic radioautographic localization of iodinated nerve growth factor bound to and internalized by PC12 cells. *J Neurosci* **3**: 631–643, 1983.
5. Walicke P and Baird A, Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. *J Neurosci* **11**: 2249–2258, 1991.
6. Maloteaux JM, Gossuin A, Pauwels P and Laduron PM, Short-term disappearance of muscarinic cell surface receptors in carbachol-induced desensitization. *FEBS Lett* **156**: 103–107, 1983.
7. Liles WC, Hunter DD, Meier KE and Nathanson NM, Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. *J Biol Chem* **261**: 5307–5313, 1986.
8. Shaw C, Van Huizen F, Cynader MS and Wilkinson M, A role for potassium channels in the regulation of cortical acetylcholine receptors in an *in-vitro* slice preparation. *Mol Brain Res* **5**: 71–83, 1989.
9. Hertel C, Coulter SJ and Perkins JP, A comparison of catecholamine-induced internalization of β-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cell. Inhibition by phenyl-arsine oxide. *J Biol Chem* **260**: 12547–12553, 1985.
10. Staehelin M and Simons P, Rapid and reversible disappearance of β-adrenergic cell surface receptors. *EMBO J* **1**: 187–190, 1982.
11. Tehrani MHJ and Barnes Jr EM, Agonist-dependent internalization of gamma-aminobutyric acid/benzodiazepine receptors in chick cortical neurons. *J Neurochem* **57**: 1307–1312, 1991.
12. Vanisberg MA, Maloteaux JM, Octave JN and Laduron PM, Rapid agonist-induced decrease of neurotensin receptors from the cell surface in rat cultured neurons. *Biochem Pharmacol* **42**: 2265–2274, 1991.
13. Mazella J, Leonard K, Chabry J, Vincent JP and Beaudet A, Binding and internalization of iodinated neurotensin in neuronal cultures from embryonic mouse brain. *Brain Res* **564**: 249–255, 1991.
14. Laduron PM and Janssen PFM, Axoplasmic transport and possible recycling of opiate receptors labelled with 3H-lofentanil. *Life Sci* **31**: 457–462, 1982.
15. Laduron PM and Janssen PFM, Retrograde axonal transport of receptor-bound opiate in the vagus and delayed accumulation in the nodose ganglion. *Brain Res* **333**: 389–392, 1985.
16. Castel MN, Malgouris C, Blanchard JC and Laduron PM, Retrograde axonal transport of neurotensin in the dopaminergic nigrostriatal pathway in the rat. *Neuroscience* **36**: 425–430, 1990.
17. Castel MN, Beaudet A and Laduron PM, Retrograde axonal transport of neurotensin in rat nigrostriatal dopaminergic neurons: modulation during aging and possible physiological role. *Biochem Pharmacol* **47**: 53–62, 1994.
18. Kessler JP and Beaudet A, Association of neurotensin binding sites with sensory and visceromotor components of the vagus nerve. *J Neurosci* **9**: 466–472, 1989.
19. Young WS, Wamsley JK, Zarbin MA and Kuhar MJ, Opioid receptors undergo axonal flow. *Science* **210**: 76–78, 1980.
20. Zarbin MA, Wamsley JK, Innis RB and Kuhar MJ, Cholescystokinin receptors: presence and axonal flow in the rat vagus nerve. *Life Sci* **29**: 697–705, 1981.
21. Pastan IH and Willingham MC, Journey to the cell center: role of the receptosome. *Science* **214**: 504–509, 1981.
22. Hopkins CR, Gibson A, Shipman M and Miller K, Movement of internalized ligand–receptor complexes along a continuous endosomal reticulum. *Nature* **346**: 335–339, 1990.
23. Heuser GE and Reese TS, Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol* **57**: 315–344, 1973.
24. Parton RG, Simons K and Dotti CG, Axonal and dendritic endocytotic pathways in cultured neurons. *J Cell Biol* **119**: 123–137, 1992.
25. Sadoul JL, Mazella J, Amar S, Kitabgi P and Vincent JP, Preparation of neurotensin selectively iodinated on tyrosine-3 residue. Biological activity and binding properties on mammalian neurotensin receptors. *Biochem Biophys Res Commun* **120**: 812–819, 1984.
26. Faure MP, Gaudreau P, Shaw I, Cashman NR and Beaudet A, Synthesis of a biologically active probe for labelling neurotensin receptors, submitted.
27. Moyse E, Rostène W, Vial M, Leonard K, Mazella J, Kitabgi P, Vincent JP and Beaudet A, Distribution of neurotensin binding sites in rat brain: a light microscopic radioautographic study using monoiodo [^{125}I]-Tyr3-neurotensin. *Neuroscience* **22**: 525–536, 1987.
28. Hammond DN, Wainer BH, Tonsgard JH and Heller A, Neuronal properties of clonal hybrid cell lines derived from central cholinergic neurons. *Science* **234**: 1237–1240, 1986.
29. Faure MP, Shaw I, Gaudreau P, Cashman NR and Beaudet A, Binding and internalization of neurotensin in hybrid cell derived from septal cholinergic neurons. *Ann N Y Acad Sci* **668**: 345–347, 1992.
30. Checler F, Mazella J, Kitabgi P and Vincent JP, High-affinity receptor sites and rapid proteolytic inactivation of neurotensin in primary cultured neurons. *J Neurochem* **47**: 1742–1748, 1986.
31. Hammond DN, Lee JL, Tonsgard JH and Wainer BH, Development and characterization of clonal cell lines derived from septal cholinergic neurons. *Brain Res* **512**: 190–200, 1990.
32. Chabry J, Gaudriault G, Vincent JP and Mazella J, Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. *J Biol Chem* **268**: 17138–17144, 1993.
33. Faure MP, Kitabgi P, Cashman NR and Beaudet A,

Hybrid cells derived from septal cholinergic neurons (SN17) express and internalize neurotensin receptors, submitted.
34. Mazella J, Chabry J, Checler F, Beaudet A and Vincent JP, Neurotensin receptors in primary culture of neurons. In: *Methods in Neuroscience, Vol. 11, Receptors, Model Systems and Specific Receptors* (Ed. Conn M), pp. 334–351. Academic Press, London, 1993.
35. Brouard A, Pélaprat D, Dana C, Vial M, Lhiaubet AM and Rostène W, Mesencephalic dopaminergic neurons in primary cultures express functional neurotensin receptors. *J Neurosci* **12**: 1409–1415, 1992.
36. Palacios JM and Kuhar MJ, Neurotensin receptors are located on dopamine containing neurons in rat midbrain. *Nature* **294**: 587–589, 1981.
37. Quirion R, Chiueh CC, Everist HD and Pert A, Comparative localization of neurotensin receptors in nigrostriatal and mesolimbic dopaminergic terminals. *Brain Res* **327**: 385–389, 1985.
38. Castel MN, Woulfe J, Wang X, Laduron PM and Beaudet A, Light and electron microscopic localization of retrogradely transported neurotensin in rat nigrostrial dopaminergic neurons. *Neuroscience* **50**: 269–282, 1992.
39. Shephard VL, Intracellular pathways and mechanisms of sorting in receptor-mediated endocytosis. *Trends Pharmacol Sci* **10**: 458–462, 1989.
40. Castel MN, Faucher D, Cuiné F, Dubédat P, Boireau A and Laduron PM, Identification of intact neurotensin in the substantia nigra after its retrograde axonal transport in dopaminergic neurons. *J Neurochem* **56**: 1816–1818, 1991.
41. Peters T and Ashley CA, An artefact in radioautography due to binding of free amino acids to tissue by fixatives. *J Cell Biol* **33**: 53–60, 1967.
42. Laduron PM, Axonal transport of neuroreceptors: possible involvement in long-term memory. *Neuroscience* **22**: 767–779, 1987.
43. Tanaka K, Masu M and Nakanishi S, Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* **4**: 847–854, 1990.
44. Faure MP, Gaudriault G, Vincent JP, Dennis M and Beaudet A, Neurotensin is internalized through receptor-mediated endocytosis. *Soc Neurosci Abstracts* **19**: 238, 1993.
45. Turner JT, James-Kracke MR and Camden JM, Regulation of the neurotensin receptor and intracellular calcium mobilization in HT29 cells. *J Pharmacol Exp Ther* **253**: 1049–1056, 1990.
46. Checler F, Vincent JP and Kitabgi P, Degradation of neurotensin by rat synaptic membranes: involvement of a thermolysin-like metallo-endopeptidase (enkephalinase), angiotensin-converting enzyme and other unidentified peptidase. *J Neurochem* **41**: 375–384, 1983.
47. Barelli H, Girard F, St. Pierre S, Kitabgi P, Vincent JP and Checler F, Further characterization of a neurotensin-degrading neutral metalloendopeptidase from rat brain. *Neurochem Int* **12**: 351–359, 1988.
48. Chabry J, Checler F, Vincent JP and Mazella J, Colocalization of neurotensin receptors and of the neurotensin-degrading enzyme endopeptidase 24.16 in primary cultures of neurons. *J Neurosci* **10**: 3916–3921, 1991.
49. Laduron PM, Towards genomic pharmacology: from membranal to nuclear receptors. *Adv Drug Res* **22**: 107–148, 1992.
50. Laduron PM, Genomic pharmacology: more intracellular sites for drug action. *Biochem Pharmacol* **44**: 1233–1242, 1992.