Neurotensin internalization in targeted neurons of the central nervous system

by

Marie-Pierre Faure

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Department of Neurology and Neurosurgery
McGill University
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ABSTRACT

In order to elucidate the mechanisms of action of neuropeptides in the central nervous system, the receptors for neurotensin, a tridecapeptide whose role as a neuromediator is well established, were visualized by confocal microscopy.

Fluo-NT (Nα-fluoresceinyl thiocarbamyl (FTC)-[Glu^1]NT), a derivative of the peptide labeled with fluorescein, binds with high affinity to SF9 insect cells infected with a baculovirus which codes for the neurotensin receptor and to hybrid neuron-neuroblastomas derived from mouse septum. This fluorescent ligand also binds to neurons in basal forebrain and mesecephalic tegmentum slices which are identified immunocytochemically as being cholinergic and dopaminergic, respectively.

In all these cellular models, the binding of neurotensin induces a rapid internalization of the receptor. This internalisation is temperature-dependent and is blocked by phenylarsine oxide, an endocytosis inhibitor. Additionally, this phenomenon remains unaffected following the covalent attachment of a photoactive derivative of fluo-NT, suggesting that the process of internalization involves the entire ligand-receptor complex.

The confocal microscopic analysis of cells labeled at 4°C, reveals a uniform distribution of receptors with zones of high density. Confocal microscopic analysis of basal forebrain and mesencephalic tegmentum preparations incubated at 37°C, illustrates that the intracellular distribution of the fluorescent ligand reorganizes with time. At short time intervals (5-15 min), the labeling is distributed throughout the cytoplasm of neuronal cell bodies and their projections. At longer time intervals (30 min to 2 h), the intensity and the size of granules increase in the cell bodies but diminish in the neuropil suggesting a centripital transport of the ligand-

receptor complex. The intrastriatal injection of fluo-NT in vivo confirmed the existence of a retrograde transport of the internalized tracer. These results suggest that the internalization of neurotensin occurs both at somatodendritic and axon terminal plasma membranes; in both cases, the number of "endosomal vesicles" diminishes with time while they increase in size, finally regrouping near the cell nucleus.

This internalization and transport process could serve to abolish the peptide signal, regulate receptor numbers and/or mediate an intracellular transduction via nuclear translocation.

RESUME

Afin d'élucider les mécanismes d'action des neuropeptides dans le système nerveux central, les récepteurs de la neurotensine (NT), un tridécapeptide dont le rôle de neuromédiateur est maintenant bien établi, ont été visualisés en microscopie confocale.

La fluo-NT, un dérivé du peptide natif marqué par le FITC, se lie avec une haute affinité aux cellules SF9 infectées par un baculovirus codant pour le récepteur de la NT, et à des hybridomes neurones-neuroblastomes préparés à partir du septum de souris. Ce ligand fluorescent se lie aussi sur des neurones identifiés par immunohistochimie comme étant cholinergiques et dopaminergiques dans des tranches de proencéphale basal et de tegmentum mésencéphalique, respectivement.

Dans tous ces modèles cellulaires, la liaison peptide-recepteur induit une internalisation rapide du récepteur. Cette internalisation dépend de la température et elle est bloquée par l'oxyde de phénylarsine, un inhibiteur de l'endocytose. Elle reste par ailleurs inchangée après liaison covalente des récepteurs par un dérivé photoactivable de la fluo-NT, suggérant que le processus d'internalisation implique l'ensemble du complexe ligand-récepteur.

L'analyse par microscopie confocale des cellules marquées à 4°C révèle une distribution relativement uniforme des récepteurs. L'analyse en microscopie confocale des préparations de proencéphale basal et de tegmentum mésencéphalique respectivement, après incubation à 37°C, met en évidence une réorganisation de la distribution intracellulaire du ligand fluorescent avec le temps. A des temps courts (5 à 15 min), le marquage est concentré àl'intérieur d'éléments granulaires parsemant le cytoplasme des corps cellulaires et de leurs prolongements. A des temps plus longs (30 min à 2 h), l'intensité et la taille des granules augmentent au niveau des corps cellulaires, mais diminuent dans le

neuropile, suggérant un transport centripète du complexe ligand-récepteur. L'injection intrastriatale de fluo-NT *in vivo* confirme l'existence d'un transport rétrograde du traceur internalisé. Ces résultats suggèrent que l'internalization de la neurotensine se produit à la fois au niveau des membranes somato-dendritiques et aussi au niveau des terminaisons axonales; dans les deux cas, le nombre de "vésicules endosomales" diminuent avec le temps tandis qu'elles augmentent en taille, pour se regrouper finalement autour du noyau.

Ces processus d'internalisation et de transport pourraient être à l'origine de l'extinction du signal peptidique, de la régulation du nombre de récepteurs et/ou d'une transduction intracellulaire par translocation nucléaire.

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Patents

A marker for neurotensin receptors. Inventors: Alain Beaudet, **Marie-Pierre Faure**, Pierrette Gaudreau. Assignee: Martinex R&D and McGill University. U.S. Patent application #997,720-December 31, 1992. Europeen Patent application # 93403185.7 December 31, 1993.

CLAIM FOR ORIGINAL WORK

The results presented in this thesis represent an original contribution to the knowledge concerning the internalization of a neuropeptide by targeted neurons in the brain. A new innovative methodology was conceived and developed for neurotransmitter receptor imaging at the cellular level. This methodological approach was used for the combined visualization of neurotensin binding sites and immunocytochemical detection of tyrosine hydroxylase (TH) or choline acetyltransferase (CaTH) at the confocal microscopy level on the same brain section. This approach enabled us to elucidate the cellular and subcellular relationships between neurotensin receptors and cholinergic neurons in the rat basal forebrain as well as dopaminergic neurons in the rat mesencephalon.

Much of this work has been presented both orally and in posters at various scientific meetings and symposia including the 1991 New York Academy of Sciences Conference on Neurotensin, 1991, 1992 and 1993 meetings of The Society for Neurosciences, the 1993-94 seminar series of Anatomy department, the 1993 seminar series of the Montreal Neurological Institute noon seminar series.

Chapter 1 presents an extensive literature review pertaining to neurotensin, its receptor and internalization in general. Also, a review of work on fluorescent probes as well as confocal microscopy is presented.

Chapter 2 is a method article describing the synthesis and biochemical characterization of a new marker for neurotensin receptor: fluo-NT which has demonstrated applications in flow cytometry, receptor localization and internalization assays.

Chapter 3 illustrates and demonstrates using quantitative imaging in living brain slices that the internalization of neurotensin is receptor-mediated, time- and temperature-dependant and that this internalization process occurs centripetally

from the somato-dendritic cell membrane to the perinuclear area in basal forebrain cholinergic neurons.

Chapter 4 presents the pharmacological characterisation of NT binding sites in cholinergic neurons in cultures as well as the internalization kinetics of neurotensin in this cell line.

Chapter 5 illustrates and demonstrates that neurotensin transport towards cell center and internalization occurs at both somatodendritic and terminal membranes of dopaminergic neurons.

Chapter 6 presents a summary of the work completed on neurotensin internalization in the laboratory of Dr. Alain Beaudet and describes the different approaches used for visualization of neurotensin receptors in the rat mesencephalon.

Chapter 7 presents a summary of the results obtained in the preceding chapters and the conclusions derived from them.

MANUSCRIPTS AND AUTHORSHIP:

The following is an excerpt from the *Guidelines Concerning Thesis Preparation,*

which we are required to include in the introductory sections of any thesis to which it applies (so that external readers will be informed of Faculty regulations).

Candidates have the option, subject to approval of their departement, of including, as part of their thesis, copies of the text of a paper (s) submitted for publication, or the clearly duplicated text of a published paper (s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory.

The thesis must still conform to all requirements of the `Guidelines Concerning Thesis preparation` and should be in a literary form that is more than mere collection of manuscripts published ot to be published. The thesis must include, as seperate chapters or sections: (1) a Table of Contents, (2) a general Abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

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To my family,

CHAPTER 1: INTRODUCTION

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1.0 NEUROTENSIN

1.1 Discovery, isolation and synthesis of neurotensin

Twenty years ago Carraway and Leeman [Carraway and Leeman., 1973], in the course of purifying substance P from bovine hypothalamic extracts, detected a second, separate bioactive substance which eluted before substance P activity by ion exchange chromatography. Intravenous injection of this purified fraction of hypothalamic extracts produced cutaneous dose-dependent vasodilatation in exposed regions, particularly in the ears and areas around the face, hypotension and cyanosis in the anaesthetized rat. Early on, it was recognized that intravenous administration of this fraction increased vascular permeability [Carraway and Leeman, 1973]. This vasoactive property was used to monitor the purification procedures and led to the isolation of a new thirteen amino acids peptide. The peptide was named neurotensin on the basis of its extraction from nervous tissue and its potent hypotensive properties in the periphery.

Two years later [Carraway and Leeman, 1975], the amino acid sequence was determinated to be p-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH utilizing bioassay and radioimmunoassay techniques. This sequence was found to be chemically identical to that of intestinal NT isolated in others mammals [Kitabgi et al., 1976]. However, it showed a marked difference at the amino (N)-terminal region when compared to chicken intestinal NT and to the NT-related anurian peptide, xenopsin [Araki et al., 1973; Carraway et al., 1980; Hammer and al., 1980; Carraway et al., 1983]. On the other hand, the C-terminal region (9-13) was found to be well preserved during the evolutionary process. NT has been extracted in all classes of vertebrates including mammals [Carraway et al., 1975; Kitabgi et al., 1976; Kobayashi et al., 1977; Uhl et al, 1976; Dupont et al., 1979; Cooper et al., 1981; Kataoka et al, 1979; Manberg et al, 1982; Papadopoulos et al. 1986], amphibians and reptiles [Langer, 1979 et al.; Reinecke et al,

1980; Bhatnagar et al, 1981; Carraway et al, 1982; Goedert et al, 1984; Eldred et al., 1987], as well as in lower life forms such as in unicellular organisms and bacteria [Bhatnagar et al., 1981; Grimmelikhuijzen et al, 1981; Carraway et al., 1982; Price et al., 1982]. This preservation suggests that NT may play a fundamental, yet unknown role in cellular functions basic to animal life.

The in vitro purification and amino acid sequencing of NT permitted the preparation of synthetic material using the solid phase method [Carraway et al., 1975; Rivier et al, 1977; St.Pierre et al, 1981]. Synthetic NT and native NT are indistinguishable biochemically. The synthesis of NT made possible the development of specific anti-NT antibodies [Carraway et al., 1976a,b]. Regional tissue concentrations of NT were determinated by radioimmunoassays (RIA) [Carraway et al., 1976a,b; Uhl et al, 1976]. NT-positive cells were localized in tissues sections by immunohistochemistry [Jennes et al, 1982; Goedert et al, 1984d]. More recently, the cloning of the gene encoding NT [Dobner et al, 1987; Kislaukis et al, 1988] allowed the visualization of sites of NT synthesis using in situ hybridization histochemistry [Alexander et al, 1989; Jayaraman et al, 1990].

In mammals, RIAs performed on micropunched tissue samples and immunohistochemical studies on tissue sections revealed NT within the central nervous system (CNS). NT was also found in peripheral tissues such as gastrointestinal tract [Sundler et al., 1977; Schultzberg et al., 1980; Reinecke et al., 1980], cardiovascular system [Mashford et al, 1978; Blakburn et al., 1979], adrenal medulla [Lee et al., 1981; Rokaeus et al., 1984] and plasma [Fernstrom et al, 1982]. Ninety percent of total body NT occurs outside of the brain, mostly in the small intestine.

Interestingly, ontogenic studies have shown that the initial differentiation and development of NT-positive neurons in the rat brain occurs during fetal period (day16)

before the establishment of normal synaptic transmission, suggesting that NT-positive neurons may play a functional role in the brain development [Hara et al., 1982].

1.2 Distribution of neurotensin in central nervous system

1.2.1 Radioimmunoassay

In several mammalian species, including man, NT is distributed heterogeneously throughout the CNS. The highest concentrations of radioimmunoassayable NT in adult rat were detected in periaqueductal grey matter, amygdala, nucleus accumbens, as well as in several hypothalamic nuclei. Moderate concentrations are present in the caudate, hippocampus and globulus pallidus [Manberg et al, 1982; Uhl et al, 1976; Kataoka et al., 1979; Jennes et al, 1982; Emson et al., 1982a; Emson et al, 1982b]. Low concentrations of NT are present in the rat, cat, calf and monkey cerebellar cortex and cerebellum [Manberg et al., 1982; Uhl et al, 1976; Kataoka et al., 1979; Jennes et al., 1982; Emson et al., 1982a; Emson et al., 1982b].

Considerable variability exists in the content of radioimmunoassayable NT in analogous brain regions of several mammalian species. This may result from a number of factors including actual species variation, post mortem delays, and differences in methods of tissue extraction. Discrepancies may also be due to differential cross reactivities of antisera for RIA and/or antisera in different species [Carraway et al, 1982; Emson et al, 1982a].

1.2.2 Immunocytochemistry and in situ hybridization.

High concentrations of immunoreactive NT cell bodies were localized in the limbic regions of the forebrain [Jennes et al, 1982], and in some hypothalamic and mesencephalic nuclei in most mammalian species, including human [Uhl et al., 1976; Uhl et al, 1979a;

Bissette et al, 1984; Manberg et al, 1982, ; Emson et al., 1985].

Additionally the relative topographic distribution of neurons expressing the pro NT/NN mRNA as revealed by in situ hybridization histochemistry correlates well with that of NT-immunoreactive cell bodies [Alexander et al., 1989]. The persistence of NT/NN mRNA in some regions despite the apparent absence of NT-positive neurons suggests that in these precursor-expressing neurons, the NT/NN mRNA may be untranslated, poorly translated, or rapidly degraded in these particular regions [Alexander et al., 1989; Checler et al., 1993; Nicot et al., 1992, Nicot et al., 1994].

The telecenphalon and mesencephalon are the two brain regions mainly involved in the present work. Consequently, the present introduction to NT localization outlines only these two areas.

1.2.2.1 Telecenphalon

NT immunoreactivity is exclusively found in nerve cells, fibers and terminals. A preponderance of NT-positive cell bodies in the adult rat are located in the medial and lateral septal nuclei, nucleus of the diagonal band of Broca as well as in the bed nucleus of the stria terminalis [Jennes et al, 1982; Emson et al., 1985]. NT neurons in the ventromedial caudate-putamen contribute to the dense band of NT positive fibers concentrated along the medial aspect of the globus pallidus [Emson et al., 1985] a region which contains magnocellular cholinergic neurons [Lehman et al., 1980; Satoh et al., 1983; Saper et al., 1984]. NT-positive neuronal perikaria in the bed nucleus of the stria terminalis send axons through the stria terminalis to provide a dense innervation to the central nucleus of the amygdala. Conversely, NT-containing neurons in the central nucleus of the amygdala send reciprocal projections to the bed nucleus of the stria terminalis [Uhl et al, 1976; Uhl et al, 1979b; Roberts et al., 1982; Zahm et al., 1987].

In situ hybridization histochemistry has revealed that the pattern of distribution displayed by cells expressing the NT/NN mRNA in the basal forebrain is largely consistent with that exhibited by NT positive neurons [Alexander et al., 1989]. Thus, NT/NN mRNA-containing neurons have been detected in the anterior aspects of the piriform cortex and endopiriform cortex, nucleus accumbens, ventral pallidum, septum, bed nucleus of the stria terminalis, ventromedial caudate-putamen, and diagonal band of Broca [Alexander et al., 1989; Nicot et al., 1992; Nicot et al., 1994].

1.2.2.2 Mesencephalon

In adult rat, NT is present in both neuronal perikaria and fibers in the ventral tegmental area (VTA) [Jennes et al, 1982; Uhl et al, 1976]. The density of NT-immunoreactive neurons is greatest in the dorsal VTA, corresponding to the nucleus parabrachial pigmentosus and nucleus lineraris [Jennes et al, 1982]. In adult rat, but not in humans, the vast majority of NT-positive cells have been colocalized with cholecystokinin and dopamine in a population of neurons in the VTA, as well as in a few neurons of the medial subtantia nigra (SN) and caudal linear raphe nuclei using double labeling indirect immunofluorescence [Hokfelt et al, 1984; Seroogy et al, 1989b; Emson et al, 1985]. The bulk of NT neurons in the VTA project to the prefrontal cortex [Studler et al., 1988], although a small percentage innervate or pass through the shell of the nucleus accumbens and diagonal band of Broca [Kalivas et al., 1984; Seroogy et al., 1989b; Studler et al., 1988]. In situ hybridization studies corroborate the existence of NT/NN mRNA neurons of the parabrachial and paranigral subdivisions of the VTA area as well as the interfacicular (IF), rostral raphe, and caudal linear raphe nuclei [Jayaraman et al., 1990]. It has been postulated that NT/NN mRNA is preferentially transcribed in those parts of the VTA that projected heavily to the entorhinal cortex and amygdala [Jayaraman et al.,

1990].

NT fibers, varicosities and terminals have been localized in the ventral tegmentum where they form a dense plexus throughout the substantia nigra pars lateralis and compacta as well as in the VTA [Jennes et al. 1982; Uhl et al., 1976; Hokfelt et al., 1984; Seroogy et al., 1989b]. NT fibers were seen by fluorescence histochemistry to approximate dopaminergic perikaria and processes in the VTA and SN [Hokfelt et al., 1984]. Furthermore, Woulfe et al. [Woulfe et al., 1989], utilizing double labelling immunocytochemistry at the electron microscopic level, reported that NT terminals form synaptic contacts on dopamine perikaria and dendrites, although a greater portion of NT synapses are onto non-dopaminergic dendrites and neuronal soma. Recently, Bayer et al [Bayer et al, 1991] found that NT is concentrated within dense core vesicles in tyrosine hydroxylase (TH) immunopositive perikaria. No dual labelled NT/TH axon terminals were observed, in keeping with the paucity of local dopaminergic axons and the persistence of local NT levels following 6-OHDA destruction of midbrain dopamine neurons [Masuo et al, 1990; Zahm et al, 1989].

NT-positive cell bodies were also detected in rat midbrain periaqueductal gray, and these have been shown to send projections to the nucleus raphe magnus [Jennes et al., 1982; Beitz et al., 1982]. NT fibers are also detectable in the retrorubral field, interfascicular nucleus, rostral and caudal linear raphe nuclei, central grey, and superficial grey of the superior colliculus [Uhl et al., 1976; Jennes et al.,1982; Hokfelt et al., 1984; Seroogy et al., 1989].

1.2.3 Tracing studies

In rat brain, a major neurotensinergic pathway has been traced with injection of horseradish peroxidase (HRP), a retrograde tracer, from the endopiriform nucleus and

prepiriform cortex to the anterior olfactory nucleus and diagonal band nucleus [Inagaki et al., 1983]. Some evidence that the endoperiform and prepiriform cortical regions give rise to NT-containing projections to the mediodorsal thalamic nucleus [Inagaki et al., 1983]. NT-positive neurons from the arcuate nucleus project to the median eminence [Ibata et al., 1983; Hokfelt et al., 1984; Sawchenko et al. 1984].

Other main pathways include projection from the ventral tegmental area (VTA) to the nucleus accumbens [Kalivas et al., 1984]. Combined immunohistochemical and retrograde transport (HRP) studies have shown that NT-containing terminals in the parabrachial region of the brainstem originate from neurons of the nucleus tractus solitarius [Milner et al., 1986]. NT neurons in the lateral parabrachial nucleus of rat send projections to the central amygdaloid nucleus as shown by HRP double immunohistochemistry and lesioning studies [Yamano et al., 1988]. NT fibres in the raphe originate from neurons in both the nucleus of the solitary tract and the central grey [Beitz et al., 1983].

NT-containing neurons have also been localized in the subiculum of the hippocampus and cingulate gyrus in the adult rat, the former providing a major NT projection via the fibria/fornix to the hypothalamus and mammillary bodies as shown by retrograde transport and electrolytic lesion studies [Hara et al, 1982; Cuello et al., 1983; Sakamoto et al., 1986].

2.0 EVIDENCE OF A NEUROTRANSMITTER ROLE FOR NEUROTENSIN

NT fulfils many of the criteria of a neurotransmitter in the central nervous system (CNS) including: (1) synthesis in selective neuronal population [Carraway et al., 1992]; (2) concentration in synaptic vesicles within synaptosomes [Uhl et al., 1977b;

Kataoka et al., 1979], (3) release upon depolarization by potassium in a Ca^{++} dependent fashion [Bean et al., 1989], (4) degradation by a variety of peptidases [Checler et al., 1982]; (5) binding to specific high affinity binding sites with pharmacological characteristics of functional receptors [Uhl et al., 1976; Young et al., 1979; Quirion et al., 1982].

2.1 Intra-neuronal biosynthesis

NT is known to be cleaved from a larger precursor molecule synthesized in the perikarion [Kahn et al, 1980; Jennes et al, 1982]. The enhancement of NT immunoreactivity in neuronal perikaria subsequent to treatment with the axonal transport-inhibiting drug colchicine [Jennes et al., 1982] implies a rapid transport of NT out of the cell body immediately subsequent to, or in the course of, its post-translational release from the precursor molecule. In situ hybridization histochemistry has demonstrated the presence of the messenger RNA encoding the NT precursor molecule in neuronal cell bodies within the CNS [Alexander et al., 1989; Jayaraman et al., 1990] thereby confirming the existence of an intra-neuronal biosynthesis.

2.1.1 Neurotensin/Neuromedin N gene

The cDNA encoding the precursor for NT and one of its biologically active co-derivatives, neuromedin N (NN) was cloned by Dobner in 1987 [Dobner et al., 1987]. The rat gene consists of four exons and three introns spanning 10.2 kilobases. Comparison of the deduced amino acid sequences reveals 76% homology among species [Kislaukis et al., 1988]. The NT and NN coding domains (120-169 residues) are localized to exon 4 at the C-terminal extremity of the NT precursor molecule [Dobner et al., 1987; Kislaukis et al., 1988]. Exon 1 encodes the signal peptide, while exon 2 and exon 3 encode amino acids 24-44 and 45-119 of the precursor, respectively [Kislaukis et al, 1988].

Furthermore, Kislaukis identified several regulatory elements present at a 200 bp region of the 5' flanking sequence such as: (1) an AP1 site, (2) two cyclic AMP response elements (CRE) and (3) a glucocorticoid response elements [Kislaukis et al., 1988].

2.1.2 Neurotensin/Neuromedin N precursor

Many neuroendocrine peptide precursor proteins encode multiple biologically active peptides, and the NT precursor shares this common feature. The overall structure of the precursor is highly conserved between species [Dobner et al., 1987]. The coding domains for NT and a structurally related six amino acid peptide neuromedin N (NN) are tandemly positioned near the carboxy terminus of the precursor protein bound and separated by lys-arg basic aminoacid pairs. The paired base residues flanking the NT and NN coding domains are likely to comprise recognition signals for cleavage by trypsin-like processing enzymes [Steiner et al., 1992]. Furthermore, Dobner and coworkers reported a NN-like sequence occurring in the middle of the precursor and bordered by paired arginyl residues at its N-terminal domains [Dobner et al., 1987] which has been shown to be cleaved in rat brain [DeNadai et al., 1989]. Pepsin-related enzymes have been implicated in the cleavage of the precursor at Lys-arg dibasic sites [Carraway et al., 1987]. ß-turns within the three-dimensional structure of the precursor molecule presumably render dibasic sites susceptible to enzymatic cleavage. The existence of ß-turns at dibasic and monobasic sites upstream from NT and NN within the rat precursor molecule is compatible with the possibility of multiple maturation cleavages [Carraway et al., 1992].

The amino terminal portion of the precursor contains a stretch of hydrophobic amino acids characteristic of the signal peptides that precede most secreted proteins [Dobner et al., 1992]. NT processing was also found to be tissue specific, with large molecular forms being more prevalent in adrenal gland and intestine than in brain.

2.2 In vitro release of neurotensin

NT has been shown to be concentrated in synaptosomes [Kataoka et al, 1979], and to be released upon depolarization by potassium [Bean et al, 1989]. The release of NT is calcium dependent and it is also stimulated by dibutryl cyclic AMP, suggesting the involvement of an adenylate cyclase system [Maeda et al., 1981].

2.3 Enzymatic inactivation

NT undergoes rapid degradation by peptidases after exposure in vitro to both soluble and particulate fractions of a variety of brain tissue preparations [Dupont et al., 1978; Martins et al., 1980; Emson et al., 1982; McDermott et al., 1982; Checler et al., 1982]. Emson et al suggested that NT was degraded at the level of Pro^{10} [Emson et al., 1982]. However, later studies employing HPLC of NT degradation fragments revealed that the degradation is mediated by three inactivating cleavages of the molecule [McDermott et al., 1982; Checler et al., 1984]. The nature of the resulting metabolites was ascertained by HPLC, amino acid analysis and RIA using both C- and N- terminal-directed antibodies.

In vitro studies on the mechanisms of NT inactivation in various membrane preparations or cell cultures [Dupont et al., 1978; McDermott et al., 1982; Checler et al., 1983; Checler et al., 1985] led to the detection of several peptidases that can be divided into 2 groups: (1) peptidases that are responsible for primary cleavages of the parent peptide, leading to degradation products totally devoid of biological activity (i.e proline endopeptidase and endopeptidase 24-11, 24-15, and 24-16), and (2) enzymes that are involved in the secondary processing of NT degradation products that cannot be considered as inactivating peptidases, such as aminopeptidase(s), postproline

dipeptidylaminopeptidase [Checler et al., 1988]

The primary hydrolytic cleavages occur at the Arg^{8}-Arg^{9}, Pro^{10}-Tyr^{11}, and Tyr^{11}-Ile^{12} peptidyl bonds [Checler et al., 1984]. This hydrolysis is inhibited by captopril and by the metal chelating agent 1,10 phenanthroline [Erdos et al., 1977]. Different peptidases are responsible for each of those cleavages [Orlowski et al., 1983; Checler et al., 1986].

The hydrolysis of the Tyr^{11}-Ile^{12} is mediated by metalloendopeptidase 24.11 also known as enkephalinase [Matsas et al., 1983]. Accordingly, the specific enkephalinase inhibitor, thiorphan [Roques et al., 1980], and the specific ACE inhibitor captopril [Ondetti et al., 1977], prevent the formation of NT 1-11. The endopeptidase 24.11 and proline endopeptidease contribute to the hydrolysis of NT at Pro^{10}-Tyr^{11} [Checler et al., 1982; Checler et al., 1984].

The cleavage of the Arg^{8}-Arg^{9} bond is mediated by the endopeptidase 24.15 [Orlowski et al., 1983; Checler et al., 1986a,b,c]. The neurotensin analogs [D-Tyr^{11}] and [D-Phe^{11}] neurotensin resist to the degradation by brain peptidases in vitro [Checler et al., 1985] and in vivo [Jolicoeur, 1984]. Both endopeptidase 24.11 and 24.15 act at the C-terminal end of NT which sustains the whole biological activity of this peptide [Kitabgi et al., 1977]. There exists a regionalization of NT degradation mechanism in the CNS. Davis et al [Davis et al., 1992], in rat brain slices, clearly demonstrated that although endopeptidase 24.15 appeared of major importance in all brain regions studied, additional contribution of angiotensin-converting enzyme and endopeptidase 24.11 were markedly elevated in the caudate putamen compared to hippocampus and nucleus accumbens [Davis et al., 1992].

Furthermore, Barelli et al [Barelli, 1988; Barelli, 1992] detected and purified a metalloendopeptidase called 3.4.24.16. Biochemical studies have shown endopeptidase

3.4.24.16 (EP 24.16) to be mainly responsible, in concert with endopeptidase 24.11, for the primary cleavage of the NT molecule at the Pro^{10}-Tyr^{11} site [Barelli et al., 1988]. The EP 24.16 is the only peptidase present in all the neurotensiceptive neurons [Checler et al, 1988; Chabry et al., 1990]. Biochemical studies revealed the existence of both a membrane-associated and a cytosoluble form of EP 24.16 [Barelli et al., 1988; Checler et al., 1988], these results are in conformity with those of immunoelectromicroscopical studies [Woulfe et al., 1992] which have shown the presence of "hot spots" of membrane immunostaining for EP 24.16 on neuronal perikaria and dendrites.

3.0 NEUROTENSIN RECEPTORS

3.1 General characteristics of neurotensin binding sites

The central and peripheral pharmacological effects of NT are mediated through the activation of specific receptors. The characteristics of which (NT-R) have been analyzed in a variety of tissues and cell lines of peripheral and central origin (for review, see Kitabgi et al.) [Kitabgi et al., 1985] : brain homogenates, synaptosomes, synaptic membranes, brain tissue sections, N1E-115 neuroblastoma cells, NG108-15 hybrid cells, HT29 human colonic adenocarcinoma cells and mouse primary neuronal cultures [Kitabgi, et al. 1977; Uhl et al., 1977a,c; Young et al., 1981; Goedert et al., 1984c; Nakagawa et al., 1984; Poustis et al., 1984; Mills et al., 1988; Turner et al., 1990; Mazella et al., 1991]. Specific binding sites have been characterized in the CNS using radioreceptor binding assays on membrane preparations from rat, mouse and human neuronal tissues [Lazarus et al., 1977; Kitabgi et al., 1977; Uhl et al., 1977a,b; Young et al., 1981; Vincent et al., 1992].

Several reports have suggested the existence of a single class of high affinity receptors [Kitabgi et al., 1980; Goedert et al,1986; Kanba et al., 1986], whereas other

investigators have described two NT binding sites [Sadoul et al., 1984b]. High affinity binding site (NT^H or NT_2 binding sites) are characterized by a high affinity (0.13nM) and a low capacity depending of the sources. On the basis of binding studies using subcellular fractionation (to discriminate between neuronal and non-neuronal elements), high affinity receptors were shown to be associated with neurons whereas low affinity receptors were present on non neuronal cells [Schotte et al., 1987]. Low-affinity binding sites (NT^L or NT_1 acceptor sites) in murine brain [Schotte et al., 1986; Kitabgi et al., 1987], are characterized by a low affinity (2.4nM) and a high capacity. Levocabastine, a known histamine H1 antagonist (RO 50 54 7:(-)-3S-[1(cis),3α,4ß]-1-[cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenyl-4-piperidine monochlororydrate carboxylic acid) has been shown to inhibit NT binding to low affinity binding sites without affecting binding to the high affinity site in rat, mouse, and hamster brain but has no effect in guinea pig, cat, dog, or human brain [Schotte et al., 1986; Kitabgi et al., 1987].

The recent synthesis of metabolically stable NT analogues with high affinity for NT receptors [Lugrin et al., 1991], and of non peptidic NT antagonists [Gully et al., 1993; Snider et al., 1993] should provide useful tools for addressing questions regarding the possible existence of receptor subtypes.

In development, as well as at different ontogenic stages, regional and subcellular localizations of NT1 and NT2 sites have been observed, using either in vitro autoradiography [Kiyama et al., 1987; Palacios et al., 1988] and membrane binding assays in rat brain [Schotte et al., 1987]. The high affinity NT receptor is expressed early during the development and is different in terms of molecular structure from the receptor present in the adult brain [Vincent et al., 1991]. The NT1 acceptor sites are not detected in rat brain before day 10 of postnatal life [Schotte et al, 1988]. The functional significance of this so-called "acceptor" site remains to be established.

Attempt at purifying NT receptors in brain mouse membrane and cultured cell preparations originally employed either photoaffinity coupling of rat synaptic membranes with ^{125}I-labelled azidobezoyl [Trp11]NT or by cross-linking 125I-[Trp11]NT to the membrane preparation using succmyl suberate [Mazella et al., 1985; Ahmad et al., 1987]. Analysis of these covalently labelled receptors using SDS-PAGE revealed two bands suggesting that the NT receptors are present in cell membranes either as a heterodimer made up of two noncovalently-linked polypeptide chains, or as a single polypeptide chain of a 51 kilodalton protein, the 49 kilodalton protein being derived from its proteolysis [Mazella et al., 1985]. Recently, Mazella et al. and Miyamoto-Lee et al. [Mazella and al, 1989] have purified a high affinity NT receptor by affinity chromatography from bovine and mouse brain, and characterized a 100kDa polypeptide which, upon proteolysis yielded the two previously identified subunits [Mazella et al, 1993].

3.2 Neurotensin receptor gene

Tanaka and co-workers [Tanaka et al., 1990] deduced the amino acid sequence of the rat high affinity NT binding site from the cloned cDNA. Thus, the cloning of the NT receptor has rendered possible a more complete elucidation of its structural and functional characteristics. Analysis of the deduce of amino acid sequence revealed that the NT receptor is composed of 424 residues. The primary sequence has a predicted molecular weight of 47,052.

More recently, a cDNA encoding human NT-R was isolated by screening a human colon adenocarcinoma cell line (HT-29) cDNA expression library using a radioligand binding strategy [Vita et al., 1993]. The rat cloned NT-R of 424 amino acids and the human of 418 amino acids. Both comprise seven putative transmembrane domains and belong to the family of G-protein coupled receptors. The amino acid sequence of the

human NT-R is 84% identical to that of the rat NT-R.

3.3 Neurotensin signal transduction

Several studies have shown that NT-R coupled to a variety of second messenger cascade systems [Goedert et al., 1984; Battaini et al., 1985; Snider et al., 1986; Kanba et al, 1987; Amar et al, 1987; Turner et al., 1990] including: 1) inositol phosphate turnover, 2) cGMP formation, 3) cAMP inhibition and 4) NT receptor phosphorylation

3.3.1 Inositol phosphate turnover

The effect of NT on the stimulation of inositol phosphate production varies from one cell line to the other. Amar et al [Amar et al., 1987] and Kanba et al [Kanba et al., 1987] have shown that in the murine neuroblastoma cell line N1E115, the coupling of the NT receptor to phospholipase C is partially mediated by a G protein that can be inhibited by pertussis toxin [Amar et al., 1987]. In the presence of lithium ions, which block inositol monophosphate hydrolysis, NT produces, a rapid and transient stimulation of inositol triphosphate and inositol biphosphate levels and a slower increase of the inositol monophosphate concentration [Amar et al., 1987]. In HT29 cell, NT is also able to stimulate phosphatidylinositol turnover without changing the intracellular levels of cAMP and cGMP [Tanaka et al., 1990].

Goedert et al [Goedert et al., 1984b] showed in whole rat brain slices that NT stimulated inositol phosphate formation and that there was a good correlation between the magnitude of NT stimulated phosphatidyl inositol hydrolysis and the number of specific NT binding sites in various brain regions [Gilbert et al., 1984].

In a Chinese hamster ovary cell line (CHO) transfected with the cDNA encoding for the cloned rat NT-R, NT also stimulates the production of inositol phosphate

[Hermans et al., 1992; Yamada et al., 1993]. Moreover, preincubation of transfected cells with pertusis toxin did not inhibit the agonist-induced inositol phosphate formation indicating that the stably expressed NT receptor was functionally coupled to phospholipase C through a pertusis toxin -insensitive G-protein mechanism [Hermans et al., 1992].

3.3.2 cGMP formation

In neuroblastoma N1E115 cells, activation of high affinity NT-R results in an increase in intracellular cGMP concentration. This effect is calcium dependent and leads to a maximal cGMP stimulation of 10 fold over basal levels [Gilbert et al., 1984b; Amar et al., 1985].

3.3.3 cAMP inhibition

A parallel 20 to 30% decrease of the cAMP basal level is also detected [Amar et al., 1985]. This inhibitory effect of NT is much easier to study after stimulation of cAMP by prostaglandins E [Bozou et al., 1986]. Under these conditions, NT inhibits cAMP production by 50 to 55 % [Bozou et al., 1986].

3.3.4 Effect of NT on protein phosphorylation

NT has been shown to play a role in signal transduction pathways mostly by mobilizing calcium stores following inositol phosphate formation. This has been attributed to subsequent events in protein phosphorylation [Cain et al., 1988]. Fewer studies have been performed aimed at characterizing the actions of NT at the phosphoprotein level. The activation of protein kinase C by DAG may lead to phosphorylation and inactivation of receptors that are coupled to the inositol phospholipid cycle in rat hippocampal slice

[Labarca et al., 1984]. Cain et al [Cain et al., 1988] demonstrated in rat striatal synaptosomes that NT stimulates the in vivo phosphorylation of three proteins of molecular mass 50kDa, 72kDa and 76kDa. Cain and Nemeroff [Cain et al., 1991] studied the effects of NT-sensitive protein phosphorylation in slices of rodent caudate nucleus. They found changes in three Ca^{2+}-dependent phosphoprotein- 56kDa, 43 kDa and 36kDa. Changes were also seen in the 43 kDa protein with cAMP treatment. The 43 kDa protein was tentatively identified as the _ subunit of pyruvate dehydrogenase. Kasckow et al [Kasckow et al., 1991] investigated the effects of NT on calcium/calmodulin-dependent protein phosphorylation in rat neostriatal slices.

3.4 Regulation of NT receptor sensitivity

Chronic exposure of neurons to neurotransmitter agonists acting on cell surface receptors results in a loss of neuronal responsiveness to the transmitter, a phenomenon known as desensitization [Maloteaux et al., 1983; Gilbert et al., 1988]. Desensitization may be achieved either by uncoupling the receptor from its associated G protein and/or effector or by decreasing the density of cell surface receptors. The loss of specific cell surface receptors is referred to as down-regulation [Gilbert et al., 1988]. Loss of binding sites may be due to their migration into the cell interior (internalization) [Maloteaux et al.,, 1983]. Internalized receptors may be recycled back to the cell surface or degraded [Vanisberg et al., 1991; Maloteaux et al., 1983].

The molecular events responsible for NT-R desensitization have not yet been defined precisely in the literature [Vanisberg et al., 1991]. The regulation of NT-R was originally described in HT 29 cells [Turner et al., 1990] and later in primary neuronal cultures as well as other cell types in culture (e.i. N1E-115)[Gilbert et al.,, 1988].

Vanisberg et al [Vanisberg et al., 1991] showed that 60% of NT-R had

disappeared from rat embryonic neuronal cell less than 10 min after agonist preincubation. This corresponded to a reduction of the Bmax value without change in the binding affinity. This could be explained either by an intracellular receptor sequestration or by receptor degradation after internalization (true down-regulation). This group [Vanisberg et al., 1991] also observed that after a longer radioactive NT exposure, 60 min, the binding of radioactive NT, in rat neuronal culture, was irreversible, which is compatible with the sequestration of labelled NT in membrane compartments or vesicles from which it cannot diffuse.

Richelson et al [Richelson et al., 1992] showed that under conditions in which NT degradation was prevented, short time preincubation of neuroblastoma clone N1E-115 with NT decreases receptor-mediated cyclic GMP formation in cells subsequently stimulated with NT. Desensitization of NT receptor-mediated cyclic GMP formation in this clone was reversible, but resensitization was a slower process than desensitization. Thus, recovery of NT-induced cyclic GMP production, to 90 to 100% of control levels was observed following incubation of the desensitized cells in the absence of NT for a minimum of 10 min subsequent to NT stimulation.

Chabry et al. [Chabry et al., 1993] by sodium dodecylsulfate polyacrylamide gel electrophoresis analysis in denaturing conditions revealed that three different protein subunits of 50, 60 and 100 kDa were covalently labelled at 37°C with a radioreactive analogue of NT. The 50 and 60 kDa subunits remained radiolabeled when NT internalization [Chabry et al., 1993] was blocked whereas the specific labelling of the 100 kDa protein was abolished. These results suggested that NT-induced internalization of the 50 and 60 kDa subunits initially present on the cell surface triggers insertion of the 100 kDa subunit into the membrane from an intracellular compartment. Subcellular fractionation experiments have shown that, in the absence of NT, the 100kDa protein is

located in an intracellular vesicular fraction of neurons [Chabry et al., 1993].

3.5 Neurotensin receptor distribution in CNS

The distribution of high affinity NT binding sites was first examined using radioreceptor assays in membrane preparations from dissected specimens [Lazarus et al., 1977; Uhl et al., 1977a] and later analyzed in frozen brain sections using in vitro "dry" radioautographic techniques [Young et al., 1979; Young et al, 1981; Quirion et al., 1982; Sadoul et al., 1984c; Sarrieau et al., 1985; Hervé et al., 1986].

The autoradiographic distribution of brain NT-binding sites has been reported for various species including rat [Uhl et al., 1977a; Lazarus et al., 1977; Young et al., 1979; Young et al., 1981; Quirion et al., 1982; Moyse et al., 1987], pigeon [Brauth et al., 1986], calf [Mills et al., 1988], cat [Goedert et al., 1984], guinea pig, monkey [Kohler et al, 1985; Quirion et al., 1987] and human [Sadoul et al., 1984b; Sarrieau et al., 1985, Kanba et al, 1986; Quirion et al., 1987; Goedert et al., 1986].

In all species examined, the distribution of specific high affinity binding sites was heterogenous. High density sites were found in the zona compacta of the SN and the VTA, as well as in the dopamine terminal fields of the olfactory tubercules and frontal cortex [Young et al., 1981]. High concentrations of NT binding sites were also detected in the lateral septum, the diagonal band of Broca, the caudate putamen, the amygdala, the dentate gyrus, the anterior dorsal nucleus of the thalamus, the suprachiasmatic nucleus of the hypothalamus, the medial habenula, and the zona incerta [Young et al., 1979; Quirion et al., 1982; Uhl et al., 1982; Moyse et al., 1987]. Low concentrations of NT binding sites were found in the subtantia innominata, and the nucleus basalis [Moyse et al., 1987].

The relative topographic patterns of distribution of NT-positive neurons and NT binding sites in the CNS are, to a large extent, comparable [Uhl et al., 1982]. However, in

several brain regions, a lack of anatomical correspondence between presumptive sites of NT release and sites of action were observed [Emson et al., 1985]. These exceptions are the cortex and striatum [Emson et al., 1985]. In cortex, NT levels are low and receptor density is high [Lazarus et al., 1977]. In contrast, in the striatum, NT concentrations are high and receptor levels are low [Goedert et al., 1984a].

3.5.1 Telencephalon

The distribution of NT binding sites in the basal forebrain corresponds relatively well with the localization of NT-containing fibers and cells in these areas. The ^{125}I-NT/or [^{3}H]NT labelling was selectively localized over neuronal perikaria, in the islands of Calleja, septofimbrial nucleus, vertical and horizontal limbs of the diagonal band of Broca and subtantia inominata [Moyse et al., 1987]. NT binding sites densities are also apparent in the medial septum, olfactory bulb, bed nucleus of the stria terminalis, and in the olfactory tubercule [Young et al., 1981; Quirion et al., 1982; Emson et al., 1985; Moyse et al., 1987]. NT binding sites are selectively concentrated over magnocellular cholinergic neurons in the diagonal band of Broca and nucleus basalis of Meynert which, in the rat, is located long the medial edge of the globus pallidus [Szigethy et al., 1989].

In the rat caudate putamen, NT binding sites are concentrated dorsolateraly, in the region where only low amounts of NT are present [Quirion et al., 1982; Zahm et al., 1987]. Labelling in the striatum spared traversing fibers of the internal capsule in this region but did not appear to coincide with striosomal compartmentation [Moyse et al., 1987]. Furthermore, studies combining 6-OHDA lesioning, kainic acid lesioning and cortical ablation indicated that more than 50% of NT binding sites in the rat striatum were localized to intrinsic neurons, 30% to dopaminergic terminals and 20% on cortical nerve fibers [Goedert et al., 1984c]. In the human forebrain, the highest densities of NT binding

sites were observed in the caudate and claustrum with lower levels in the putamen [Quirion et al., 1987]. In the rat, monkey and human amygdala, moderate to high levels of NT receptors were found in the medial, basomedial, and cortical nuclei [Quirion et al., 1982; Sarrieau et al., 1985; Quirion et al., 1987]. A low density was observed in the rat nucleus accumbens, a region concentrated in NT [Quirion et al., 1982].

NT binding sites in the hippocampus are concentrated over the lateral enthorinal area, pre and parasubiculum, ventral subiculum, and induseum griseum [Young et al., 1981; Quirion et al., 1982; Kohler et al., 1985; Moyse et al., 1987; Kohler et al., 1987].

3.5.2 Mesencephalon

The distribution of high concentrations of NT binding sites, in both rat and cat VTA, corresponds to the localization of NT-containing fiber terminals and perikaria in these areas [Palacios et al., 1981]. The demonstration that the antihistamine, levocabastine exerts a negligible effect on ^{125}I-NT binding in these areas suggests that these binding sites correspond to the functional, high affinity receptor [Szigethy et al., 1989].

Lesioning of the VTA and SN with the neurotoxin 6-OHDA produced a substantial diminution of NT receptors [Palacios et al., 1981; Quirion et al., 1982; Goedert et al., 1984c; Hervé et al., 1986] Moreover, using combined tyrosine hydroxylase (TH) immunocytochemistry and ^{125}I-NT autoradiography on adjacent light microscopic sections, Szigethy and Beaudet [Szigethy et al., 1989] found that more than 80% of all dopamine cells were in register with intense ^{125}I-NT labelling indicating that an important proportion of neurotensin binding sites in the VTA and SN were associated with dopaminergic neurons. Furthermore, approximately 20% of NT binding associated with dopaminergic neurons was found by electron microscopy to be intraneuronal, perhaps

corresponding to sites of NT receptor synthesis. The remainder of binding sites were located diffusely over the membrane of perikaria and dendrites, and showed no predilection for synaptic specializations. This observation lead to the suggestion that NT, in the nigra and VTA, acts in a paracrine fashion to regulate the tonic activity of dopamine neurons [Szighetty et al., 1989].

Mesencephalic NT binding sites have also been detected in the periaqueductal grey, dorsal raphe nucleus, and the superficial gray of the superior colliculus [Young et al., 1981; Quirion et al., 1982; Moyse et al., 1987].

3.6 Structure-activity relationships

Structure-activity studies in a variety of bioassay and radioreceptor assay systems have allowed the characterization of the structural requirements for the NT-NT receptor interaction. These studies have established that the N-terminal fragments of the NT molecule, NT 1-6, NT 1-8, NT 1-11 are inactive while the C terminal peptides, NT 8-13 and NT 9-13 are biologically active [Carraway et al, 1976; Kitabgi et al., 1977; Jolicoeur et al., 1984]. The C-terminal hexapeptide portion of NT 8-13 contains all the structural requirements for receptor binding and activation [Kitabgi et al., 1980; St. Pierre et al., 1981; Kitabgi et al., 1984]. Interestingly, all the natural NT-related peptides share strong structural homologies with neurotensin (8-13) and it is therefore not surprising that neuromedin N and xenospin interact with NT receptors [Checler et al., 1982; Checler et al., 1986]. This region (8-13) is well conserved in chicken, frog and guinea-pig NT while several differences exist in the congeners isolated from the stomach of the opossum or the skin of the turkey [Kitabgi et al., 1977; Jolicoeur et al., 1984; Li et al., 1984].

3.6.1 Neuromedin N

Neuromedin N (NN), a hexapeptide, originally extracted from porcine spinal cord, shares a 4 amino acid homology with the C-terminal region of NT [Minamino et al., 1984]. NN and NT are synthesized from a common 170 amino acid molecular precursor wherein they are separated by a single pair of lysine-arginine basic residues. Moreover, their synthesis appears to be differentially regulated in the CNS, as they show a regionally differential distribution [Carraway et al., 1987] (see section 1.2). and levels of NN are lower than NT [DeNadai et al., 1989]. Furthermore, the two peptides are co-released from hypothalamic slices [Kitabgi et al, 1990]. NN has been shown to exert effects similar to NT including causing contraction of guinea pig ileum, producing analgesia [Carraway et al., 1973; Coquerel et al., 1988].

NN is more active that NT in increasing spontaneous motor activity after injection into the ventral tegmental area (VTA) [Kalivas et al., 1986]. On the other hand, NN does not produce a hypothermic response after injection into the VTA and is less active than NT at inhibiting dopamine-induced behavioral hyperactivity [Kalivas et al., 1986].

NN has been shown to compete with NT for occupancy of NT receptors, but has a lower affinity for these binding sites then NT [Kitabgi et al., 1985; Gilbert et al., 1986].

NN is degraded much more rapidly that NT by membrane peptidases which yield fragments devoid of biological activity [Checler et al., 1986d]. This enhanced vulnerability is due to the fact that the N-terminal region of NN is less protected than NT and is therefore more susceptible to enzyme degradation. Accordingly, the affinity of NN for NT receptors was shown to be potentiated (5-fold) in the presence of metallopeptidase inhibitors [Checler et al., 1986d].

4.0 EVIDENCES FOR A NEUROMODULATOR ROLE FOR NEUROTENSIN

4.1 Neurotensin effects on different systems.

Over the last fifteen years, a growing number of neuropeptides have been shown to play an important role in the neuromodulation of classical neurotransmitter function in the mammalian nervous system [Stinus et al., 1980; Skirboll et al. 1981; Bannon et al., 1983; Kalivas et al., 1985]. Biochemical, electrophysiological, anatomical and behavioral studies suggest that NT may be interacting with a variety of central transmitter specific systems [Palacios et al., 1981; Kalivas et al., 1984; Quirion et al., 1985; Nemeroff et al., 1985]. In particular NT has been shown to interact with cholinergic, dopaminergic, serotonergic, as well as noradrenergic, neuronal systems [Goedert et al.,1982; Ibata et al., 1983; Goedert et al., 1984d; Alexander et al.,1984]

There is scant evidence in the literature supporting an interaction between basal forebrain cholinergic neurons and NT [Moyse et al., 1987; Wenk et al., 1989; Lapchak et al., 1989; Lapchak et al., 1991; Szigethy et al., 1990] whereas the interaction between striatal dopamine and NT has been extensively documented [Kalivas et al., 1984; Studler et al., 1988; Bean et al., 1989].

4.2 Selective association of neurotensin receptors with cholinergic neurons.

Receptor autoradiographic studies in the rat have demonstrated the presence of high densities of specific high-affinity NT binding sites (levocabastine insensitive) in the basal forebrain and cerebral cortex [Moyse et al., 1987]. These are areas which exhibit rich cholinergic innervation [Kasa et al, 1986]. Cholinergic projections from the basal forebrain give rise to the laminar distribution of choline acetyltransferase (ChAT)-positive staining in the frontal, parietal, and occipital cortex. The majority of ChAT staining is scattered throughout layers 1 and 5 in the frontal cortex, layers 1-3 and 5 in the parietal cortex, and layer 4 in the occipital cortex [Eckenstein et al., 1988]. In both human and rat

basal forebrain, NT-R have been shown to be associated with a subset of acetylcholineresterase-positive cells in the nucleus basalis magnocellularis (NBM), subtantia innominata, medial septum, and nucleus of the diagonal band of Broca [Szigethy et al., 1989]. Furthermore, recently Szigethy et al have demonstrated that acetylcholinesterase-positive neurons of the rat and human basal forebrain are in register with NT receptors [Szigethy et al., 1987; Szigethy et al., 1989; Szigethy et al., 1990].

The first evidence for an interaction between NT and acetylcholine (ACh) in the CNS comes from the work of Malthe-Sorenssen et al [Malthe-Sorenssen et al., 1978] who have shown that intracerebroventricular injection of NT in rats decreases the content of ACh in the parietal cortex, without altering that of the frontal cortex. Lapchak et al [Lapchak et al., 1990] demonstrated a local regulation of Ach release by NT at the level of the neuron terminals in the frontal and parietal cortex. In the frontal cortex, NT decreased evoked ACh release in a TTX-insensitive manner (tetradotoxin) suggesting a direct action of NT on frontal cholinergic terminals [Lapchak et al., 1990].

Cholinergic neurons in the medial septum, horizontal limb of the diagonal band, and nucleus basalis have been shown to be markedly decreased in patients with Alzheimer's disease, with a concomitant loss of memory function [Whitehouse et al., 1981; Wilcok et al., 1982; Pearson et al., 1983; Arendt et al., 1983]. Indeed, NT levels have been shown to be significantly decreased in the septal nucleus of such patients [Ferrier et al., 1983].

4.3 Selective association of neurotensin receptors with dopaminergic neurons

4.3.1 Dopamine mesencephalic neurons

The A10 dopamine cell group is mainly located in the VTA. The VTA was documented in the original description by Tsai [Tsai et al.,1925] as a histologically distinct

group of neurons in the ventral mesencephalon that, in the sagittal plane, appear triangular in shape.

The VTA is subdivided into dorsal and ventral compartments, referred to as the nucleus parabrachialis pigmentosus and nucleus paranigralis, respectively. Lateral to the A10 region is the substantia nigra (SN), which contain the A9 dopaminergic (DA) neurons. The DA perikaria are located along the dorsal edge of the SN in the pars compacta (SNC), whereas their dendritic fields extend ventrally into the pars reticulata (SNR). Dorsal and caudal to the SN is a cluster of DA neurons referred to as the A8 cell group by Dahlstrom and Fuxe [Dahlstrom et al., 1964] which is localized in the retrorubral field.

The projection fields of midbrain DA neurons are topographically organized. The dopamine ascending projections are subdivided into the mesocorticolimbic system emanating from the A10 region, and the nigrostriatal system originating from the A9 neuron group. The more medial cells in the VTA project most densely to limbic and cortical regions, while the lateral neurons in the SNC project more exclusively to the striatum [Fallon et al., 1978; Beckstead et al., 1979].

4.3.2. Neurotensin pathways

Neuroanatomically, NT is closely associated with DA neurons at the level of both their cell bodies in the midbrain and their terminal fields in the mesencephalon [Levant, 1988]. High concentrations of NT are detected in the SN, the VTA, the nucleus accumbens (NAS) and the caudate nucleus (see chapter 1.2)

4.3.3 Anatomical association of neurotensin receptors and dopaminergic neurons

An interaction of NT with midbrain (DA) neurons has been suggested by that central NT systems are in proximity to DA systems [Palacios et al., 1981; Quirion, et al. 1985; Ward et al., 1988; Woulfe et al., 1989; Szigethy et al., 1989]. Anatomically, NT is colocalized with DA in neurons of the arcuate nucleus (A12), as well as with DA in neurons of the VTA (A10) and SN (A9)[Seroogy et al., 1987].

The report by Palacios and Kuhar [Palacios et al., 1981] of a large depletion of NT-R in the SNC following local 6-hydroxydopamine injection was the first to provide evidence for a direct effect of NT on midbrain DA cells. The association of NT-R with midbrain DA neurons was later confirmed in the rat [Quirion et al., 1985; Hervé et al., 1986] and in the human brain where a decrease in nigral NT-R densities in post-mortem specimens from patients with Parkinson's disease was observed [Sadoul et al., 1984; Uhl et al., 1984; Chinaglia et al., 1990]. Using a combined autoradiographic and immunohistochemical approach, Beaudet's group later demonstrated that the vast majority of TH immunopositive neurons in the SN (>95%) and the VTA (>80%) were endowed with NT-R [Szigethy et al., 1989]. In turn, virtually all neurons harbouring NT-R in the ventral midbrain were found to be TH immunopositive. Using electron microscopic autoradiography, this group also showed that NT-R were associated with both the perikaria and dendrites of mesencephalic DA cells and that they were more or less uniformly distributed along the entire plasma membrane and not particularly concentrated at the level of synaptic contacts [Dana et al., 1989, Brouard et al., 1992].

4.3.4 Neuromodulation of dopaminergic neurons by neurotensin.

4.3.4.1 Modulation of dopamine receptors

Nemeroff et al [Nemeroff et al., 1983] originally observed that centrally administered NT exerts a neuroleptic-like action, which probably was related to an

interaction of the peptide with the ascending DA pathways, especially the mesolimbic systems [Nemeroff et al., 1983; Nemeroff et al., 1986]. In 1984, Fuxe et al [Fuxe et al., 1984] proposed that modulation by NT of DA bursting to D2 receptors explain NT's neuroleptic-like activity. In studies performed on in vitro membrane preparations of the rat basal ganglia and post-mortem human caudate-putamen, NT was found to reduce the binding affinity of [^{3}H]-n-propylnorapomorphine ([^{3}H]NPNA), a DA agonist, in the striatum and NAS [Agnati et al., 1983; Von Euler et al., 1990; Ahlenius et al., 1992]. Moreover, intraventricular injections of NT produced a dose dependent (0.3-3nM), monophasic reduction of specific [^{3}H]NPNA binding within the caudate putamen, the NAS, and the olfactory tubercule, confirming that NT, reduces the *in vivo* the affinity of D2 receptors [Fuxe et al., 1992]. The same group has recently reported that the reduction in DA binding caused by NT is apparently independent of a G protein mechanism [Von Euler et al., 1991]. The electro-physiological studies of Shi et al [Shi et al., 1992] showed that the decrease in sensitivity of D2 receptors by NT is abolished by blocking PKA.

Recently, an alternative hypothesis has been proposed by Adachi et al [Adachi et al., 1990]. These authors have postulate that the co-release of NT and DA leads to the formation of a NT-DA complex, thereby reducing the ability of DA to bind to the D2 receptor. This hypothesis was ruled out by Nouel et al [Nouel et al., 1992] who showed that the Kd for the synaptosomal uptake of [^{3}H]dopamine was not modified in the presence of NT. NT binding was also not modified by the addition of DA. Furthermore the nuclear magnetic resonance spectrum of NT was not modified by the addition of DA.

4.3.4.2 Electrophysiological effects

The electrophysiological actions of NT in the mesencephalon are again reminiscent of those of systematically administered antipsychotic drugs.

Numerous *in vivo* and *in vitro* electrophysiological studies have shown NT to produce an increase in the firing rate of DA neurons in both the zona compacta of the SN and VTA [Andrade et al., 1981; Bunney et al., 1991; Shi et al., 1992]. Microiontophoresis of high concentration of NT (>100nM) caused an increase in the firing rate of few DA neurons in vivo [Andrade et al., 1981; Shi et al., 1987]. *In vitro* experiments indicated that the excitatory effect of NT on DA neurons occurred at higher concentrations (>10nM) of the peptide than those needed to produce the neuromodualtory effects [Pozza et al., 1988; Shi et al., 1991]. Its persistence during DA receptor blockade by sulpiride suggests that this effect was not entirely mediated by an attenuation of the inhibition induced by endogenously released DA [Shi et al., 1991]. At even higher concentration of NT (>100nM), a sudden cessation of cell activity preceded by an increase in firing rate was observed, followed by cessation of measurable activity again [Pozza et al., 1988; Seutin et al., 1989]. Whether this effect of NT is due to depolarization, inactivation or to a toxic effect of the peptide at high concentrations remains to be determined. Investigations conducted in high magnesium and low calcium-containing media support the view that the NT-induced excitation occurs via a calcium-dependent mechanism [Seutin et al., 1989]. This mechanism may involve either a decrease or an increase in membrane conductance [Stowe et al., 1991].

An inhibitory effect of NT on DA neurons was less frequently observed. In some studies this inhibitory effect seemed to be indirect since it disappeared when synaptic transmission was blocked [Stowe et al., 1991; Shi et al., 1992].

The electrophysiological effects of acute and chronic neuroleptic treatment on the electrical activities of midbrain dopaminergic neurons have been extensively studied [Bunney et al., 1991]. Most clinically efficacious antipsychotics increase the firing rates of DA neurons in both the VTA and the SNC [Bunney et al., 1973]; repeated administration

induces "depolarization bloc" of DA neurons in these same two regions [Chiodo et al., 1983; White et al., 1983; Grace et al., 1983; Bunney et al., 1991]. Both application of haloperidol, sulpiride, and cis-flupenthixol have demonstrated an attenuation of DA-induced inhibition in the SN.

4.3.4.4 Effects on dopamine synthesis turnover, and release

Intracerebroventricular injection (ICV) of NT, like antipsychotic drugs, increases the concentration of the major DA metabolites in the NAS, olfactory tubercule and nucleus caudatus [Widerlov et al., 1982].

Biochemical studies, in fact, have shown that in vivo and in vitro applications of NT into the rat SN increase DA synthesis and release in DAergic terminal areas [Nemeroff et al., 1983; Faggin et al., 1989]. This increase in DA release is associated with increased motor activity. Local injection of NT into the rat VTA also increases DA turnover in the NAS, olfactory tubercules, and the diagonal band of Broca, as reflected by the increase in the concentration of the major DA metabolites, homovanilic acid (HVA) and dihydroxyphenylacetic acid (DOPAC). Local application of DA into the VTA also stimulates DA release in the striatum and the NAS [Meyers et al., 1984; Blaha et al., 1990]. However, it also causes autoreceptor activation and thereby decreases tyrosine hydroxylase activity [Reches et al., 1983].

Injection of NT into the rat NAS, like parenterally applied of neuroleptics, blocks the increased locomotion and rearing produced by the indirect DA agonists methylphenidate, cocaine [Nemeroff, 1983] and d-amphetamine [Ervin et al., 1981], drugs which are believed to act by increasing the synaptic availability of DA [Nemeroff et al., 1983]. Thus providing further evidence for NT's inhibition on DA release.

NT also blocks the apparent rewarding electrical self stimulation observed in rats

with electrodes placed in the VTA [Luttinger et al., 1981]. Thus, it appears that the behavioral effects induced by increase DA turnover and release in the NAS and VTA, are also blocked by NT administration.

4.3.5 Dopamine modulation of neurotensin neurons.

Govoni et al [Govoni et al., 1980], have reported that administration of the DA antagonist, haloperidol, elevates NT levels in the striatum and NAS. This was the first evidence that NT systems might be subjected to dopamine regulation. In the rat, acute and chronic (8 months) treatment with clinically efficacious antipsychotic drugs (e.g. haloperidol, chlorpromazine) have consistently resulted in an increase in NT concentrations in the NAS and striatum while phenothiazines (e.g. promazine, promethazine which are not antipsychotics) did not produce this effect [Govoni et al., 1980; Radke et al., 1989; Merchant et al., 1989]. A variety of studies using selective neurotransmitter receptor antagonists have shown that these neurochemical alterations in NT concentrations were exerted predominantly through D1 receptors [Merchant et al., 1989; Nemeroff et al., 1992].

Neurotensinergic neurons located in distinct regions of the forebrain respond differentially to typical versus atypical neuroleptics. Thus, NT neurons in the shell of NAS appear to be sensitive to most antipsychotic drugs, whereas those in the dorsolateral striatum respond only to typical neuroleptics (which are known to cause acute extrapyramidal motor side effects such as dystonia) [Nemeroff et al., 1992].

Several investigators have examined the influence of DA on the modulation of prepro-NT/NN mRNA content or gene expression in various brain regions [Merchant et al., 1991; Augood et al., 1991; Levant et al., 1992; Bean et al., 1992; Augood et al., 1992]

These studies have indicated that after a single injection of haloperidol (a typical

neuroleptic) and clozapine (an atypical neuroleptic), the prepro NT/ NN mRNA expression is significantly increased in the shell compartment of the NAS [Alheid et al., 1988; Wadworth et al., 1990; Merchant et al., 1993]

Bean et al [Bean et al., 1989] have shown that the reserpine induced depletion of striatal DA was accompanied by a dose-and time-dependent increase in striatal NT concentrations, thereby suggesting that DA afferents may control the release of striatal NT and/or the synthesis of NT within striatal NT cells. This study also revealed that these mechanisms (i.e.striatal changes) resulted from a decrease in NT release into the striatal extracellular fluid.

4.3.6 Clinical implications

It has been suggested that NT may represent an endogenous neuroleptic [Nemeroff, 1980]. Indeed, NT demonstrates pharmacological, behavioral and electrophysiological effects that are similar in many respects to those of clinically efficacious antypsychotics. Furthermore, it was showed in a subgroup of untreated schizophrenic patients with reduced NT levels that control of psychotic symptoms by neuroleptic therapy occurred in parallel with a return to normal of cerebrospinal fluid NT levels [Nemeroff et al., 1980; Lindstrom et al., 1988].

Patients who suffer from Parkinson's disease show a significant cell loss in the substantia nigra pars compacta, which is associated with a significant reduction in striatal dopamine levels [Agid et al., 1987]. Moreover, these abnormalities have been reported to include several neuropeptidergic systems; in human brain, prominent losses in striatal and nigral NT receptors were demonstrated in idiopathic Parkinson's disease [Uhl et al. 1976; Chinaglia, et al. 1990].

5.0 NEUROTENSIN INTERNALIZATION IN NEURONS

5.1 Classical internalization model in non-polarized epithelial cells.

5.1.1 Receptor-mediated endocytosis

Receptor-mediated endocytosis is a general process by which a wide variety of ligands are taken up with high specificity and affinity into cells and are enclosed in membrane-bound vesicles and taken into the cell [Dautry-Varsat et al., 1984]. Internalization of ligands is a rapid, energy-dependent process which has been studied in detail by a large number of investigators using subcellular fractionation techniques as well as morphological methods such as radioautography and immunocytochemistry [Posner et al., 1981; Bergeron, et al. 1985].

The initial event consists in the binding of the ligand to specific receptors on the cell surface. Then, ligand-receptor complexes move to specialized regions of the membrane where they complex form clusters. These specialized regions have been referred to as bristle-coated pits [Roth et al., 1964; Pearse, et al 1975]; the bristle coated pits are composed of three clathrin heavy chains (180 kDa protein), three clathrin light chains (33-36 kDa protein) [Brodsky et al., 1988] and a protein complex composed of 100, 50, and 16 kDa components termed accessory proteins or adaptors, (AP-2), [Ahle, et al. 1988; Keen, et al. 1990] that appear to interact with the cytoplasmic domains of various receptors [Glickman et al., 1989].

The movements of proteins embedded in the membranes can be visualized by fluorescence micrography [Willingham et al., 1980; Willingham et al., 1982; Hollenberg, et al. 1986] or video microscopy and measured by photobleaching methods [Poo et al., 1974]. The clustering of receptor-ligand complexes involved an initial very rapid (i.e. less than 1 s) microclustering of the receptors into dimers or many polymers (10-mers), followed by a less rapid (seconds to tens of seconds) aggregation at the level of coated

pits.

The ligand-receptor complexes are then internalized into the cell along the following classical itinerary: clathrin-coated (or non-coated) vesicles first bud from the plasma membrane and fuse together to become early endosomes of 150 to 500 nm in diameter [Wileman et al., 1985; Gruenberg et al., 1989; Pearse, et al. 1990; Rodman et al., 1990]. In early endosomes, many ligands dissociate and are sorted away from their receptors, which in some cases return to the cell surface (receptor recycling) [Dautry-Varsat et al., 1984; Mellman, et al. 1983]. Some endocytosed material is then transferred from early endosomes to late endosomes or pre-lysosomes [Schmid et al., 1988; Stoorvogel et al., 1991] which have been described as multivesicular compartments containing receptors, ligands, and other solutes destined for degradation [Griffiths et al., 1988]. Lysosomes provide the terminal compartment of the endocytic pathway: where proteins and other macromolecules are degraded by acid hydrolases.

The endosomal and lysosomal compartments contain an ATP-dependent proton pump [Galloway et al., 1984; Brown et al., 1987] and the receptor-ligand complexes encounter a progressively lower pH as they move from coated/or non-coated vesicles through the endocytotic pathway [Tycko et al., 1982; Yamashiro et al., 1987; Fuchs, et al. 1989].

5.1.2 The endosomes

The term "endosome", which was first described by Stochem in 1969 [Stochem et al., 1969], referred to the intracellular nonlysosomal components involved in the internalization and concentration of exogenous substance by cells. Endosomes are morphologically very heterogenous structures, appearing as vesicles of a variety of sizes (0.1-5μm diam) or even as tubules [Helenius et al., 1983; Marsh et al., 1986].

In addition to their differences in intra luminal pH, early endosomes and late endosomes, can be distinguished by their constituent membrane proteins, their characteristic density when separated by centrifugation, and their location in the cell.

Early and late endosomes may be distinguished by their content in small GTP binding proteins of the Rab family [Mayorga et al., 1989; Bourne et al., 1991; Takai, et al. 1992; Downward et al., 1992; Hall, et al. 1992; Boroch, et al. 1993]. The first two members were called Ypt1 [Gallwitz et al., 1988] and Sec4p [Saminen et al., 1987]. It is postulated that each transport vehicle in the cell has its own Rab protein. In keeping with this view more than 30 members of the Rab family have been identified [Touchot et al., 1987; Zahraoui et al., 1989; Chavrier et al., 1990; Valencia et al., 1991; Elferink et al., 1992]. The mechanism of action of YPT1/SEC4/Rab proteins is not yet understood. The current working hypothesis assumes that these proteins undergo a complex cycle of events, moving with the carrier vesicle from the donor compartment to the acceptor compartment and from there back to the starting point to initiate a new cycle [Mayorga et al., 1989; Gound et al., 1991; Pfeffer et al., 1992; Zerial et al., 1993].

Two of these GTP-binding proteins, Rab5 and Rab4, have shown to be associated with early endosomes. Rab5 regulates endocytosis from coated pits at the plasma membrane to early endosomes [Bucci et al., 1992] and Rab4 controls recycling from early endosome to the cell surface [Van der Sluijs et al., 1992]. By contrast, Rab7 regulates late events in endocytosis, possibly the transport from early endosome to late endosome or between the late endosome and the vacuole [Chavrier et al., 1990; Gorvel et al., 1991].

5.1.3 Lysosomes

Lysosomes serve as the major digestive compartment of mammalian cells. They are responsible for the degradation of extracellular material internalized by endocytosis.

Lysosomes are morphologically very heterogenous structures, appearing as vesicles of a variety of sizes (0.1- 5μm, diam), and mainly containning degradative enymes such as acid hydrolases

At early stage in their biosynthesis, soluble lysosomal enzymes become selectively phosphorylated on specific mannose residues. These receptors, found the trans-Golgi reticulum [Kornfeld et al., 1989] are responsible for their targeting to lysosomes. Two distinct mannose 6-phosphate receptors have been identified: the first, discovered by Sahagian et al [Sahagian et al., 1981], is now general referred to as the cation-independent mannose 6-phosphate receptor and has been shown to be identical to the type II insulin second receptor is a homodimer of 461KD, which is now referred to as the cation-dependent 6-phosphate receptor.

5.1.4 Cellular traffic

The road map describing the traffic system is characteristic for each cell type. Traffic from one station to another along the roads in the cell is carried out by membrane vehicles, in the form of either vesicles [Rothman et al., 1992] or tubules [Klausner et al., 1992].

Current debate about the mechanism by which internalized material is transported through the endocytic pathway centres around two models. In the first or "vesicle shuttle" model, early endosomes, late endosomes and lysosomes exist as stable, functionally distinct organelles [Helenius et al., 1983]. Newly formed endocytotic vesicles fuse and deliver their contents to the early endosomes. Next, vesicles budding from the early endosomes recycle to the cell surface after delivering part of the compartment's content into the late endosomes. Movement from the late endosomes to lysosomes occur in a

similar fashion. In the second or "maturation" model, newly internalized vesicles fuse together to form nascent early endosomes, which by a remodelling process, mature progressively into late endosomes and then lysosomes [Stoorvogel et al., 1991; Murphy et al., 1993; Dunn et al., 1992]. Accordingly, the identity of any individual endocytic compartment is transient [Griffits et al., 1991]. The two models, have in common the interaction between stable and transient populations of vesicles.

Hopkins et al [Hopkins et al., 1990], using different fluorescent tags to follow by video microscopy both transferrin and epidermal growth factor receptors in living epidermoid carcinoma cells, suggested that the early part of the endocytotic pathway corresponds in fact to a network of interconnected tubules.

Taken together these observations suggest that endosomes and lysosomes are highly plastic organelles [Knapp et al., 1990] and that in vivo they are connected to each other by fusion and fission events [Parton et al., 1992].

5.2 Internalization in a highly polarized cell, the neuron.

Over the past 10 years, substantial progress has been made in our understanding of receptor-mediated endocytosis in eukaryotic cells [Bergeron, 1979; Posner, 1981; Hubbard, 1989]. However, most of the studies carried out so far were on epithelial and neuroendocrine cell lines [Hogue-Angeletti, 1982; Morel, 1987; Pow, 1991]. Only very few studies have so far been carried out on neurons [Parton, 1992], or cells which present a certain degree of cellular polarity [Dotti, 1988; Ginzburg, 1991].

5.2.1 The neuron polarity

The neuron is a highly polarized cell [Dotti, 1988; Dotti, 1990]. Both the axonal and somatodendritic domains, which determine the gross polarity of the neuron,

the axonal and somatodendritic domains, which determine the gross polarity of the neuron, are polarized further into numerous sub-specialized regions. Kobayashi et al have postulated the existence of a functional barrier at the axonal Hillock/initial segment which prevents the intermixing of membrane constituents between axonal and somatodendritic domains [Kobayashi et al., 1977]. The dendritic and axonal processes differ from each other in morphology [Bartlett et al., 1984], in their capacity for protein synthesis [Davis et al., 1992], in some of the molecular constituents of their cytoskeleton [Caceres et al., 1984; Shaw et al., 1985] and in their plasma membrane [Lindsey et al., 1985; Banker et al., 1988]. Dendrites are relatively short, highly branched, tapering processes that contain polyribosomes and synthesize proteins. Axons are long, thin, and relatively uniform in diameter, lack polyribosomes, and do not synthesize proteins [Lindsley et al., 1987]. Morphological differences between dendrites and axons appears to be dependent in part on microtubule assembly [Yamada et al., 1970; Bass et al., 1988] which in turn depends upon cytoskeleton molecular composition. Brain microtubules are composed primarily of tubulin and MT-associated proteins (MAPs), a heterogeneous group of proteins that copolymerize with tubulin and promote microtubule assembly in vitro [Dotti et al., 1987]. Biochemical studies have demonstrated that while the high molecular weight MAP2 is localized in the cell body and dendrites, Tau MAP is localized in the axons [Matus et al., 1986; Peng et al., 1986]. In keeping with these observations, Litman et al [Litman, 1993] have demonstrated in rat primary cell cultures, employing in situ hybridization techniques, that tubulin mRNA is confined to cell bodies, and that MAP2 mRNA extends into dendrites whereas tau mRNA enters the proximal portion of the axon.

5.2.2 Endocytotic pathways in neurons

The traffic problems in neurons are similar to those in epithelial cells [Simons et

al., 1993]. Both cell types have to ship membrane components to at least two domains of the cell surface. In neurons these two domains are the axonal and the somatodendritic plasma membrane. Recent studies have revealed a fence localized in the axonal hillock region blocking lateral diffusion in both leaflets of the axolemma [Kobayashi et al., 1992]. The analogy to epithelial cells has been carried out one step further in that viral glycoproteins known to be transported to the apical and basolateral plasma membrane domains in MDCK cells are sorted to the axonal and somatodendritic domains, respectively [Dotti et al, 1990].

Parton and collaborators in 1992 studied the overall organisation of neuronal endocytic pathways for the first time in hippocampal neurons cultures [Parton et al., 1992]. These neurons have endocytotic pathways from both the presynapse and the postsynapse [Parton et al., 1992]. The dendrites and cell body seem to contain extensive network of tubular early endosomes which receive endocytosed markers from the somatodendritic domains. The circuit in the presynaptic terminal possibly includes synaptic vesicles. The prevailing view is that the synaptic vesicles are formed from axonal early endosomes and then taken up as transmitters for delivery to the synaptic cleft where the synaptic vesicles are stimulated to fuse [Kelly et al., 1990; Kelly et al., 1993]. One specific Rab protein, Rab3a, is thought to be involved in synaptic vesicle docking and fusion [Mizoguchi et al., 1990; Fisher von Mollard et al., 1992].

Late endosomes (possessing rab7 protein) and lysosomes were shown to be predominately located in the cell body and in the proximal segments of the dendrites [Parton, 1992]. Multivesicular bodies appear to be the major structures mediating transport of endocytosis markers between the nerve terminal and the cell body [Parton, 1992].

Recently, evidence has been obtained for a transcytotic route in hippocampal

neurons [Ikonen et al., 1993]. Transcytosis in neurons would provide an interesting intracellular route for direct communication between presynaptic and postsynaptic elements of the synapse [Simons et al., 1993].

5.2.3 Axonal transport

A cellular function unique to neurons is axonal transport of proteins. This transport has been shown to occur bidirectionally [Grafstein et al., 1980]. Newly synthesized proteins and lipids move from the cell body to the nerve terminals in two distinct components: a fast component (1-10 mm/hour) and a slow component (1-2 mm/day). Transport in each of these components appears to involve different cytoskeletal elements [Bisby et al., 1976; Grafstein et al., 1980; Tytell et al., 1981].

The axoplasmic transport of receptors makes it possible for signal molecules to be transported retrogradely from nerve terminal to the cell body. Peripheral nerves represent an ideal model to study these dynamic processes because ligature experiments can be easily performed and the accumulation of axonal radiolabeled constituents may be detected on both sides of the ligature. Several receptors were found to undergo axonal transport in peripheral axons as exemplified in build up of autoradiographically detectable binding sites: opiate [Young et al., 1980; Laduron et al., 1982], ß-adrenergic [Zarbin et al., 1983], nicotinic [Ninkovic et al., 1983], cholecystokinin [Zarbin et al., 1981], and NT receptors [Kessler et al., 1989].

If axoplasmic transport of receptors is now well established in peripheral nerves [Laduron et al., 1987; Laduron et al., 1990], there is still little evidence available for this process in the central nervous system. Castel et al [Castel, 1990] provided the first evidence for the existence of an axonal retrograde transport of a neuropeptide in the brain.

5.2.4 Internalization in neurons

Biochemical and/or autoradiographic studies have provided evidence for receptor-induced internalization of muscarinic [Maloteaux et al., 1983; Liles et al., 1986; Shaw et al., 1989; Maloteaux et al., 1994], ß-adrenergic [Staehelin et al., 1982; Hertel et al., 1985; Goldstein et al., 1985], GABA-benzodiazepine [Tehrani, 1991], a variety of neuropeptide receptors [Laduron et al., 1992; Maloteaux et al, 1994] growth factors [Seiler et al., 1984]; nerve growth factor [Stoeckel et al., 1975], and basic fibroblast growth factor [Walicke et al., 1991; Eckenstein et al., 1994]. However, all of these studies were carried out either in non-neuronal cells or in neuronal and/or glial cells in culture so that it is still unclear whether the same type of internalization process is operative in the brain.

Taken together these studies mainly demonstrated that internalization involves receptor regulatory processes as well as desensitization. Furthermore, little is known of the sequence of events triggered by neurotransmitter internalization or of the subcellular compartments that subserve it.

5.3 Neurotensin internalization in neurons

Over the last decade, the desensitization of NT-R has been attributed to intracellular sequestration and recycling of the receptor in primary neuronal and non neuronal cell cultures, implying that the NT-R regulation may be due to the ligand-induced internalization of NT.

The internalization of NT-R was first described in HT29, a colonic cell line by Turner in 1990 [Turner et al., 1990] and more recently by Vanisberg et al [Vanisberg et al., 1991] in embryonic brain cultures. They demonstrated that the agonist induced a rapid decrease in the number of NT-R from the cell surface which corresponded to an internalization process.

By using a combination of biochemical and light microscopic radioautographic approaches, Mazella et al [Mazella et al., 1991] have demonstrated the internalization of radiolabeled NT in mouse embryonic cultures.

Castel et al [Castel et al., 1990] showed that injection of radiolabeled NT into the rat neostriatum induced its retrograde transport towards the ipisilateral substantia nigra and adjacent ventral tegmental area. They also showed that this transport was dependent upon receptor-dependent internalization of peptide at the level of striatal DA nerve terminals. Moreover this group showed an increase in TH expression in the SN after NT retrograde transport [Burgevin et al., 1992].

Several studies suggest that the interaction of NT with its receptor is followed by a ligand-induced internalization of the peptide receptor complex. High resolution and electron microscopic radioautograms demonstrate the "pile up" and the presence of intra-perikarial NT binding sites within the ligated vagus nerve and in neurons of the CNS, respectively. Recently Hermans et al [Hermans et al., 1994] showed that after association with Chinese hamster ovary (CHO) cells expressing the rat NT-R, tritiated NT was rapidly internalized.

The molecular determinants/signals for the internalization of the NT-R are still unknown. The transfected cells expressing the wild type or mutagenized forms of the NT-R provide an interesting pharmacological tool for the identification of receptor domains specifically involved in the physiological response and in the receptor internalization process [Hermans 1994, personal communication].

Mutational analysis of the NT-R using truncations of the carboxyl terminus revealed that the intracellular region between residues 1092 and 1126 is important for rapid internalization [Hermans, 1994 personal communication]. This is in accordance with the fact that the cytoplasmic region of receptors and even a restricted domain of the C-

terminal region seems to play a key role in the internalization process.

6.0 FLUORESCENT ANALOGS OF PEPTIDES

6.1 The nature and measurement of fluorescence

Fluorescence is the property of certain molecules (fluorophores) to absorb light at one wavelength and emit light at another. The incident light "excites" the molecule to a higher level of vibrational energy: as the molecule returns to its original state it emits photons. Excitation and emission occur over a band of wavelengths referred to as the "excitation spectrum " and the "emission spectrum". Both spectra have a maximum which is used to describe the properties of the molecule, and to define the optimum conditions for detection of fluorescence.

6.2 Available fluorophores

Fluorophores or fluorochromes are fluorescent dyes or probes that are added to molecules to make them fluorescent. Fluorophores are chosen or synthesized for particular applications based on several criteria such as absorption and emission spectra, extinction coefficient, quantum yield, and chemical reactivity. Some of the most common fluorophores are Fluorescein (FITC), Bodipy which have a fluorescence spectra in the green and Phycoerythrin Cyanine 3.18, Texas red, Allophycocyanin which have a fluorescence emission spectra in the red.

6.3 Fluorescent peptides

The vast majority of current studies on the pharmacology and anatomical localization of neurotransmitter receptors in mammalian CNS rely on the use of isotopicaly labelled ligands [Yamamura et al., 1985; Snowhill et al., 1986; Conn et al.,

1993]. However, the radiation hazard of isotopes has recently led to the development of alternative labels.

Fluorescence techniques are uniquely suitable for probing living neurons because of their sensitivity, specificity and non toxicity. Over the last twenty years, there have also been a few attempts at using ligands tagged with fluorescent markers. Thus, fluorescent derivatives of alpha and beta adrenergic agonists have been tested for *in vivo* labelling of their respective receptors in rat brain [Atlas et al., 1978; Hess et al., 1979; Correa et al., 1980]. Fluorescent-tagged benzodiazepines R07-1986 and RO15-1788 have been applied to the pharmacological characterization of GABA-A receptor complexes in nerve cell membrane preparations [Havunjian et al., 1990; McCabe et al., 1990]. Enkephalin and naltrindole derivatives have been used for visualizing mu and delta opiate receptors in rat brain sections [Correa et al., 1980] neuroblastoma cells [Hazum et al., 1980] and primary neurons in culture [Elde et al., 1989], respectively. Bursztajn et al [Bursztajn et al., 1984] have resorted to a fluorescent acetylcholine analog for the biochemical characterization of nicotinic receptor channels in chick myotubes. Finally, a variety of fluorescent D1 and D2 receptor ligands have been developed for biochemical detection and anatomical visualization of dopamine receptors in sections of rat neostriatum [Barton et al., 1991; Ariano et al., 1991].

The use of fluorescent receptor probes has nonetheless remained somewhat limited, in part because of the notorious difficulty in tagging receptor ligands with fluorophores without affecting their biological properties [Taylor et al., 1980], but also and mainly because of the lack of sensitivity of available fluorescence detection methods for measuring relatively small number of cell surface molecules. This latter limitation has of late largely been overcome through the development of highly sensitive detection and visualization techniques such confocal laser scanning microscopy, which offers the

required degree of sensitivity for pharmacological characterization of receptors and their localization at the cellular level, respectively [Brakenhoff et al., 1989].

6.4 Confocal laser scanning microscopy

6.4.1 Introduction

Confocal scanning optical microscopy, arguably the most significant advance in biological light microscopy in this decade, enables one to obtain quantitative non-invasive optical sections through labelled biological specimens, virtually free from out-of focus blur. A set of these optical sections collected at a series of focal levels though an object constitutes a three-dimensional image which may then be processed digitally for display as a computer reconstruction, a stereo pair or an animation sequence.

The term confocal is derived from the fact that both the objective and the collector lenses are simultaneously focused on the same point of interest [Wilson et al., 1984]. Compared with traditional methods, confocal microscopy in combination with digital image processing has the following advantages: (1) a truly three-dimensional reconstruction of the object is obtained, (2) the specimen remains intact, (3) a higher resolution than fluorescence microscope can be obtained, (4) the scanning process is automated.

6.4.2 History

The principle of confocal microscopy was first described by Minsky [Minsky et al., 1961], but it was not until Egger and Petran reported its successful application to unstained neuronal tissue that the potential usefulness of this technique in the neurosciences became apparent [Egger et al., 1967]. The ability of confocal optics to provide high-magnification images of high resolution has made the confocal laser

microscope an important tool for the study of a wide variety of nervous tissue preparations [Fine et al., 1988].

6.4.3 CLSM : basic principles

In confocal laser scanning microscopy an excitatory point illumination source with a wavelength in the ultraviolet range (e.g. 488nm) is reflected from a dichroic mirror (an optical gate, reflecting light higher energy/shorter wavelength and passing light of lower energy/longer wavelength) into a scanning device (mirrors or vibrating crystal) which moves in a scan in an *x-y* plane. [Minsky et al., 1961]

Conventional microscope objective optics focus the scanned light in the specimen; the point of focus is the first of the two related points necessary for confocal microscopy. With high numerical aperture objectives, the light hits the sample at large angles, producing a very thin plane of focus [Wilke et al., 1985].

The high energy laser light interacts with the specimen, which emits lower energy fluorescent light. This light passes back through the microscope optics, scanner, and dichroic filter to reach a simple achromatic lens that focuses it on an aperture: the pinhole. This is the second related point of focus necessary for confocal microscopy. Finally, a detector (usually a photomultiplier tube) picks up the light, and the signal is amplified and digitalized. An image is formed by electronically storing and processing the single picture elements (as pixel) from each subsequent scan across the object field and displays a composite image of the entire filed on a video monitor. A graphics computer controls all mechanical and electronic operations as well as image storage,processing, and presentation. This arrangement permits not only collection of serial sections for 3D reconstruction, but also vertical scan *(Z*-plane) through the sections [Wilson et al., 1984; Brakenhoff et al., 1988]. Because of the narrow plane of focus, the resulting images are

cleaned of out-of-focus elements [Deitch et al., 1991; Staugaitis et al., 1991].

Typical scanning times for one plane acquisition run from 1/2 second for a coarse low resolution scan to up to 5 minutes for a scan on which frame averaging is done to create high resolution images.

With high magnification, high numerical aperture objectives, resolution of 200nm structures can be achieved within the plane of the optical section.

An image can be produced either by moving the light source over the object [Davidovits, 1971; Aslund, 1983; Wlike, 1984] or by scanning the object through the very narrow light path (by moving the specimen stage) [Brakenhoff, 1979]. Beam scanning is potentially faster, and is not influenced by specimen size and mass. Stage scanning, on the other hand, gives a uniform optical response over the whole image field (which can potentially be very large).

Images can be summed, averaged ,subtracted from or added to stored images. A wide range of digital image enhancement methods can be applied, including contrast-stretching, false-colour intensity coding, and various image convolutions leading to edge-enhancement or other forms of spatial frequency filtering.

6.5.4 Practical Applications

6.5.4.1 Removing out of focus haze in thick samples

The ability of the confocal laser scanning microscope to detect a fluorescent signal from a uniformly thin "optical section" eliminates ambiguities arising from variable thickness of specimen [Wilson, 1990]. The reduction of out-of-focus light in the confocal images makes it possible to see details within thick biological specimens with a resolution unobtainable by other means [Fine, 1988].

6.5.4.2 Three- dimensional cellular reconstruction

By collecting a number of consecutive sections, a perfectly aligned recording of the whole specimen volume can be obtained. Using this technique there is no need for mechanical sectioning, a process that destroys the specimen and produces sections without mutual alignment. Thin optical sections generated by the confocal microscope can provide an efficient alternative to microtome sectioning or *camera lucida* reconstructions of 3-D neuronal structures. The stored images contain a full 3-D representation of the specimen [Carlsson et al., 1989].

Image reconstruction from series of optical sections involve the assignment of spatial coordinates to each intensity value in the data set. In the case of confocal microscopic data, each optical section consists of a two dimensional array of digitized intensity values associated with specific x,y coordinates. Since the distance between consecutive optical sections is known, and since the optical sections are collected sequentially and are in perfect register, a z coordinate can also be assigned to each data point based on the known distance between successive optical sections. Thus each point can be assigned a specific x,y,z coordinate corresponding to a particular location in the original sample. This procedure allows for effective reconstruction a three dimensional image from the optical data set.

There are two general methods for rendering three dimensional images, both of which rely on advanced graphics computing techniques. The first method is called volume rendering: each data point in the reconstructed image represents a cubic volume element called voxel caring the intensity value associated with the original image data point. The second method is called surface rendering [Cline et al., 1988]. In this method, the three dimensional array of data points is converted into voxels which are traversed using a "marching cubes" algorithm to generate meshed polygons which define isosurfaces of

specific intensities within the original data set.

6.5.4.3 Simultaneous dual detection of two different stains

The image information can be obtained simultaneously by scanning defined area with excitating laser light containing both the 488 and 514 nm wavelengths necessary for the dual excitation, the emitted fluorescence passe through a second dichroic mirror placed just before the detector. The longer emission wavelength red light passed through directly to detector 1 and the shorter emission wavelength green was reflected to detector 2. The resulting image shows the overlay or merged image of the two channels. Since there is only one scan and since the light is split after the aperture, there is exact pixel-to-pixel register between the two channels [Klarsfeld et al., 1991].

7.0 SPECIFIC OBJECTIVES

As documented in the preceding sections, NT and its receptors have been shown to be widely and heterogeneously distributed in the mammalian CNS. NT has been proposed to neuromodulate the activity of basal forebrain cholinergic neurons as well as of midbrain DA neurons. The overall objective of the current thesis was to further characterize the events that follow the interaction of NT with its high affinity receptor on these transmitter-defined neuronal populations and in particular to document the mechanisms pertaining the subsequent internalization and intracellular mobilization of the peptide.

The first objective of the present work was to develop a specific fluorescent marker for central NT-R. The synthesis and characterization of this compound is described in **Chapter 2**.

Previous studies from our laboratory have demonstrated a selective association of ^{125}I-NT labelled receptors with a subpopulation of DA neurons of the ventral midbrain and cholinergic neurons of the basal forebrain in the rat using combined high resolution radioautography and immunohistochemistry on consecutive brain sections.

It was the second objective of the present work to examine the binding and internalization of NT in cholinergic cells in brain slices and to examine the kinetics of this process in immortalized cholinergic cell in vitro.

In **Chapter 3** we demonstrate using confocal microscopy the existence of receptor-mediated internalization and dendro-somatic transport of NT in basal forebrain cholinergic cells. In **Chapter 4** we demonstrate the presence of NT binding sites on a cholinergic hybrid cell line, SN17, and characterize the mechanisms of NT internalization.

Previous studies from our laboratory have demonstrated that NT is retrogradely transported in neurons of the substantia nigra following injection in the neostriatum

[Castel, 1992]. The objective of the **chapter 5** was to confirm that this transport was confined to DA neurons and to compare the intracellular distribution of transported material with that observed following internalization of the peptide at the somatodendritic level.

Appendix is a review of biochemical, autoradiographic and confocal microscopic studies of internalization of NT, in DA neuronal cell cultures *in vitro* and in the central nervous system *in vivo*

The present studies provide compelling evidence for receptor-mediated internalization and subsequent intracellular mobilization of NT in cholinergic and DA neuronal cells. Both biochemical and anatomical experiments support the concept of a time- and temperature-dependent entry of the peptide inside nerve cells. The present work also demonstrates an intracellular migration of internalized ligand molecules from distal process to nerve cell bodies on the one hand, and from the periphery of the cells to the perinuclear region on the other hand. The perinuclear clustering of internalized ligand molecule, observed here both *in vitro*, in *ex-vivo* and *in vivo*, suggests a long-term genomic effects of NT on its target cells.

CHAPTER 2

SYNTHESIS OF A BIOLOGICALLY ACTIVE FLUORESCENT FOR LABELLING NEUROTENSIN RECEPTORS

Marie-Pierre Faure, Pierrette Gaudreau[1], Ivan Shaw, Neil R. Cashman and Alain Beaudet

Neurobiology group, Montreal Neurological Institute, Montreal, Quebec, Canada H3A 2B4

[1] Laboratory of Neuroendocrinology, Notre-Dame Hospital Research Center, Montreal, Quebec, Canada H2L 4K8

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ABSTRACT

A fluorescent derivative of the tridecapeptide, neurotensin (NT) was synthesized with the aim of providing a new tool for the pharmacological characterization and anatomical localization of NT receptors in mammalian brain. Fluoresceinylated NT (Nα-fluoresceinyl thiocarbamyl (FTC)-[Glu^1]NT; fluo-NT) was synthesized using a solid phase methodology and purified to 99% homogeneity by preparative high pressure liquid chromatography (HPLC). Analytical HPLC, acidic and carboxypeptidase Y hydrolysis, and fast atom bombardment-mass spectroscopy confirmed that the purified compound was selectively labeled on the [Glu^1] terminus and that a single FTC moiety was coupled to each molecule of [Glu^1]NT. Flow cytometric analysis of the binding of fluo-NT to SN17 septal-neuroblastoma cells indicated that the fluorescent derivative bound neural NT receptors with an affinity comparable to that of monoiodinated NT (^{125}I-NT). Competition experiments on mouse brain membrane preparations showed fluo-NT to inhibit specific ^{125}I-NT binding with a coefficient of inhibition (K_I) virtually identical to that of the native peptide (0.67 vs 0.55nM). Conventional epifluorescent and confocal microscopic analysis of specific fluo-NT binding to sections of the rat midbrain revealed a topographic distribution of the bound fluorescent ligand similar to that previously observed with autoradiography using ^{125}I-NT. However, fluo-NT provided for markedly higher cellular resolution and enabled, in particular, the detection of hitherto unnoticed intra-cytoplasmic receptor clusters. Binding of fluo-NT to live SN17 hybrid cells indicated that the fluorescent ligand had retained its ability to internalize *in vivo* and confirmed that this internalization process was both time and temperature dependent. In sum, the present study demonstrates that fluo-NT is applicable to both the pharmacological study of NT binding sites using flow cytometry, and to the regional and

cellular localization of these sites by conventional epifluorescent and confocal microscopy.

INTRODUCTION

Studies aimed at characterizing the biochemical structure, pharmacological properties, and anatomical localization of neurotransmitter receptors in mammalian central nervous system (CNS) have so far mainly relied on the use of radioactive ligands or of antibodies raised against purified receptor proteins or their cloned sequences (1),(2),(3),(4). There have also been a few attempts at using ligands tagged with fluorescent markers. Thus, fluorescent derivatives of alpha and beta adrenergic agonists have been proposed for in vivo labelling of their respective receptors in rat brain (5),(6),(7). Fluorescent-tagged benzodiazepines R07-1986 and RO15-1788 have been applied to the pharmacological characterization of GABA-A receptor complexes in nerve cell membrane preparations (8),(9). Fluorescent enkephalin and naltrindole derivatives have been used for visualizing delta opioid receptors in neuroblastoma cells (10),(11),(12). Fluorescent _-bungarotoxin and anthroylcholine bromide have been resorted to for the characterization of peripheral nicotinic and central muscarinic receptors, respectively (13),(14). Finally, a variety of fluorescent D1 and D2 receptor ligands have been developed for the biochemical detection and anatomical visualization of dopamine receptors in sections of rat neostriatum (15),(16).

The use of fluorescent receptor probes has nonetheless remained somewhat limited, in part because of the notorious difficulty of tagging receptor ligands with fluorophores without affecting their biological properties (17), but also and mainly because of the lack of sensitivity of available fluorescence detection methods for measuring relatively small numbers of cell surface molecules. This latter limitation has of late largely been overcome through the development of highly sensitive detection and visualization techniques such as flow cytofluometry and confocal laser scanning

microscopy, which offer the required degree of sensitivity for pharmacological characterization of receptors and their localization at the cellular level, respectively (18). It is with these techniques in mind that we have developed a FTC-labelled neurotensin (NT) derivative (fluo-NT) for the selective tagging of high affinity NT receptors in mammalian brain.

Neurotensin is a tridecapeptide originally isolated from bovine hypothalamus (19) and subsequently identified throughout the CNS of a variety of species, where it has been shown to play a neuromodulator role (see (20),(21) for reviews). This role is exerted through NT's interaction with high affinity G-protein coupled receptors, known to be widely, but selectively distributed throughout the brain (22),(23),(24),(25). A second, low affinity NT binding component has also been reported in murine brain but the functional significance of this so-called "acceptor" site remains to be established (26). Recently, Tanaka et al. (27) have cloned from rat brain a cDNA sequence coding for a high affinity NT receptor. Whether this sequence codes for all high affinity NT receptors or only for one subtype of a larger receptor family remains to be determined.

In the present study, we have characterized the binding properties of fluo-NT in mouse brain membrane preparations as well as on septal neuron-neuroblastoma hybrid cells previously shown to express high affinity NT binding sites (28) using conventional binding techniques and flow cytometry, respectively. The applicability of the ligand for cellular visualization of high affinity NT binding sites was also investigated, both in frozen sections from the rat midbrain, a region previously documented to contain amongst the highest concentrations of NT binding sites in mammalian brain (22), (23), (24), (25), and in the same line of hybrid cells as used for flow cytometric studies.

MATERIALS AND METHODS

Synthesis, purification and characterization of fluo-neurotensin

N-FTC-[Glu^1]NT was synthesized by solid phase methodology (29) using a scheme based on tert-butyloxycarbonyl(t-Boc) chemistry/acid labile amino acid protection as previously described for other peptides (30),(31). After completion of the synthesis and selective deprotection of the Nα-amino group, acylation of this amine function was performed with a 6-fold excess of fluorescein isothiocyanate isomer I (FITC, Sigma, St.Louis, MO, U.S.A.). The reaction was performed at room temperature, in anhydrous N,N-dimethylformamide (DMF) containing 5% N,N-diisopropylethylamine, with gentle agitation. It proceeded to completion within 2 hours, as evaluated by a ninhydrin colorimetric test (32) which allows detection of free amino groups. The N_-fluoresceinyl thiocarbamyl (FTC)-peptide-resin intermediate was then extensively washed with DMF, dried in vacuo, and submitted to hydrogen fluoride cleavage (30),(31) to deprotect amino acid side chains and to cleave the Nα-FTC-peptide from the resin. The FTC-labeled peptide was then solubilised in trifluoriacetic acid (TFA), subjected to rotary evaporation in vacuo, and dried further on a freeze dryer. It was purified by preparative high pressure liquid chromatography (HPLC) on a Partisil ODS-3 Whatman column (10μ-particle size; 50 cm x 2.2 cm I.D.), using appropriate gradients of a binary solvent system consisting of 0.01% aqueous TFA, pH 2.9 and acetonitrile (CH_3CN)-0.01% TFA. Elution of the peptide was monitored at 214 nm. Collected fractions were readily screened by analytical HPLC using both UV (214 nm) and fluorometric detection (excitation, 338 nm; emission, 425 nm), pooled accordingly, evaporated in vacuo to remove CH_3CN and lyophilized twice. The homogeneity of Nα-FTC-[Glu^1]NT was evaluated by analytical HPLC on a Bondapak C18 column (10μ-particle size; 150 mm x 3.9 mm I.D.) using two solvents

systems (0.01% aqueous TFA , pH 2.9 and CH_3CN-0.01% TFA; 0.01M ammonium acetate, pH 6.6 and CH_3CN). [Glu^1]NT was synthesized according to the same methods as Nα-FTC-[Glu^1]NT and its HPLC profile compared to that of the fluoresceinylated peptide. The amino acid composition and peptide content of both synthetic compounds were assessed by quantitative amino acid analysis after acidic hydrolysis *in vacuo* (6N HCl, 110°C, 18h) or carboxypeptidase Y digestion (6 U/ 0.3 µmol peptide, 37°C, 48h) and phenylisothiocyanate (PITC) derivatization (33). Finally, the molecular mass of N_-FTC-[Glu^1]NT was determined by fast atom bombardment-mass spectroscopy (FAB-MS) on a Kratos MS-50 TATC instrument.

Flow cytometric analysis of fluo-NT binding to SN17 hybrid cells

Hybridoma cells SN17, produced by the fusion of mouse embryonic septal cells with murine neuroblastoma, were generously provided by Hammond (34). The cells were grown on 100mm tissue culture plates in Dubelcco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco, BRL, Burlington, Ont., Canada) in a humidified atmosphere of 90% air, 5% CO_2 at 37°C. For saturation experiments, the cells ($1x10^6$ cell/ml) were washed in PBS and centrifuged at 500g for 5 min at 4°C and resuspended to a final concentration of $4x10^6$ cells/ml. Twenty five µl aliquots of this washed preparation were then incubated with graded concentrations of fluo-NT (0.16 nM to 16 nM) in Hepes-Tris-buffer, pH 7.4 for 60 min at 4°C. Additional samples were incubated in the presence of a hundredfold excess of unlabeled NT for determination of non specific binding. At the end of the incubation, all cells were washed twice in cold buffer and centrifuged at 500g for 5 min at 4°C. They were then resuspended in 0.2 ml cold PBS and analyzed with a Beckton Dickinson Facscan flow cytometer (Mountain View Ca., U.S.A.) under 0.2W laser excitation. In most cases, 5 000 events/sample were collected in list mode fashion,

stored and analyzed by a consort 30 program. Debris and dead cells were excluded from the analysis by the conventional scatter getting method. Mean fluorescent intensity (MFI) was calculated using integrated signals (i.e. the integral of fluorescence intensity over time taken for each cell to pass through the laser beam) using a minimum of 2500 cells. This technique allowed relative fluorescence intensities to be monitored continuously against time to ensure that equilibrium between bound and free fluorescent ligand was reached under each condition.

Competitive ^{125}I-NT binding assay

Neurotensin was iodinated by the lactoperoxidase method and monoiodo-[^{125}I-Tyr^{3}]NT (^{125}I-NT) was separated on a Sephadex affinity column as previously described (35). Membranes were prepared from mouse brain homogenates (21), and protein concentrations determined by the method of Lowry et al (36) using bovine serum albumin as a standard. For competition binding experiments, homogenates (0.2mg protein/ml) were incubated for 30 min at 22^{o}C with 0,2nM ^{125}I-NT in the presence of a range of concentrations of unlabeled NT or fluo-NT. The reaction was stopped by the addition of 2 ml ice-cold buffer. The reactive mixture was then immediately filtered over Gelman-filters (Millipore, Missisauga, Ont., Canada) under vacuum using a millipore filtration apparatus. After two additional washes, the radioactivity was measured in a Picker gamma counter. The data were expressed for each concentration of cold competitor as a percentage of maximum specific binding. IC_{50} and K_I values were obtained using EBDA/LIGAND programs (37).

Binding of fluo-NT to rat midbrain sections

Adult male Sprague Dawley rats were sacrificed by decapitation and the brains

were rapidly removed, blocked in the coronal plane, and frozen at -40°C. Sections (20μm) were cut through the midbrain on a cryostat, mounted onto gelatin-coated slides, and incubated with 20nM fluo-NT at 4°C in the dark for 60 min in 50 nM Tris-HCl buffer, pH 7.4, containing 0,1% bovine serum albumin and 1μM of the peptidase inhibitor 1,10 phenanthroline (38). Additional sections were incubated in the presence of a hundredfold excess of unlabeled ligand for determination of non specific binding. At the end of the incubation, sections were rapidly washed in cold PBS (4x60 sec), rinsed in distilled H_2O, and air-dried in the dark under a cool stream of air.

Sections were initially examined on a Leica epifluorescence photomicroscope operating with a high pressure 100-W mercury lamp and the appropriate dichroic filter combination for excitation/emission of fluorescein (485/520nm). Selected fields were further scanned using a Leica confocal laser scanning microscope (CLSM) comprised of a Leica diaplan inverted microscope equipped with an argon ion laser (488 nm) with an output power of 2-50mW and a VME bus MC 68020/68881 computer system coupled to an optical disc for image storage (Leica, St.Laurent, Quebec, Canada). All image generating and processing operations were carried out using the Leica CLSM software package. The images were photographed from the monitor using a Focus system.

Confocal microscopic analysis of cholinergic hybrid cells

SN17 hybrid cells were grown to 50% confluence on 18 mm^2 polylysine-coated (100mg/ml) glass coverslips in 35mm tissue culture dishes. The cultured coverslips were then incubated with 10^{-6} M fluo-NT in the dark for 45 min at 4°C, in the presence or in the absence of a hundredfold excess of unlabeled NT for determination of non specific binding. After incubation, cells were either washed twice with cold PBS, or warmed up at 37°C for a further 45 min. Both sets of cells were then air-dried in the dark and placed on

a glass slide containing a drop of immersion oil. Confocal laser analysis of fluo-NT labeling was then performed as described above.

RESULTS

Characterization of Nα-FTC-[Glu^1]NT

Solid phase synthesis of Nα-FTC-[Glu^1]NT (fluo-NT) and [Glu^1]NT yielded compounds that could be purified to 99% homogeneity using preparative HPLC. The overall yield of the synthesis/purification procedure was 11% for fluo-NT and 14% for [Glu^1]NT. In both cases, approximately 52% of the amount of crude peptides loaded onto the column were recovered after purification. Characterization of the synthetic peptides by analytical HPLC yielded two distinct chromatographic profiles (Figure 1). The retention time of fluo-NT was almost twice that of [Glu^1]NT (28.2 vs 15.3 min) and its UV absorbance, especially at 280 nm, was 4 to 5 times greater, suggesting that an hydrophobic and aromatic moiety such as FITC was coupled to [Glu^1]NT (Figure 1). Amino acid analysis after acidic hydrolysis indicated that the amino acid composition of synthetic [Glu^1]NT and Nα-FTC-[Glu^1]NT conformed to expected theoretical values (Table 1). CPY digestion of Nα-FTC-[Glu^1]NT did not proceed to completion under the present experimental conditions, however amino acid analysis of the hydrolysate allowed us to demonstrate the presence of a lysyl residue (theoretical value, 1 residue; experimental value, 0.88 residue), confirming that the lysine in position 8 had not been modified and was not bearing a FTC moiety. Finally, FAB-MS analysis of N_-FTC-[Glu^1]NT yielded a theoretical molecular mass of 2081, indicating that a sole FTC moiety was bound to [Glu^1]NT. The lyophilised N_-FTC-[Glu^1]NT has proven stable for more than 1 year when kept at 4°C and protected from light and moisture.

Binding of fluo-NT to SN17 hybrid cells and mouse brain homogenates.

Flow cytometric analysis of fluo-NT binding to whole SN17 hybrid cells yielded a

single fluorescence peak (unimodal distribution) under both total and nonspecific binding conditions, indicating that virtually all of the clonal cells had bound the fluorescent molecule to a smiliar extent (Figure 2). However, the mean intensity of bound fluorescence was markedly lower in cells incubated with an excess of non fluorescent NT (non specific binding; Figure 2a) than in cells incubated with fluo-NT alone (total binding; Figure 2b). In both conditions, the peak was confined to a relatively narrow range, indicating that cells were labeled fairly homogeneously.

As can be seen in Figure 3, specific NT-FTC binding was saturable and reached a plateau at ligand concentrations of 11-16 nM. By contrast, binding in the presence of an excess of non fluorescent NT increased linearly with the concentration of fluorescent ligand, to reach 20% of total at concentrations of 16 nM.

Fluo-NT totally inhibited specific binding of ^{125}I-NT to mouse brain membrane preparations in a concentration-dependent manner (Figure 4). A coefficient of inhibition (K_I) of 0.67 nM and a pseudo-Hill coefficient close to unity were derived from the competition curve. These values were virtually identical to those obtained using native NT as a competitive drug (K_I=0.55 nM; Figure 4).

Binding of fluo-NT to rat brain sections.

Sections of the rat midbrain incubated with 10 nM fluo-NT exhibited discrete apple-green fluorescent labelling throughout the substantia nigra, ventral tegmental area and interfascicular nucleus (Figure 5b-e). This distribution pattern conformed to that previously described for specific ^{125}I-NT binding in the rat midbrain tegmentum using light microscopic autoradiography (22) (23) (24) (25) (Figure 5a). Fluo-NT labelling was no longer apparent in sections incubated with an excess of unlabelled NT. No autofluorescence was observed except for a few orange spots characteristic of lipofuschin

deposits.

At high magnification of either conventional epifluorescent or confocal microscopes, the label was seen to be concentrated over both neuronal perikarya and dendritic processes (Figures 5c-e). Within nerve cell bodies, the labelling characteristically took the form of granular hot spots, 0.5-2 μm in diameter, distributed throughout the cross-sectional profile (Figure 5e). By contrast, dendritic labelling appeared much more diffuse and floccular in nature (Figure 5c). Serial optical sectioning of the labelled sections in the confocal microscope revealed that the label was not confined to the cell surface but present throughout the cytoplasm of both perikarya and dendrites. Serial sectioning also indicated that the nucleus was virtually devoid of labelling.

In the substantia nigra, pars compacta and lateralis, the labelling was mainly apparent over nerve cell bodies (Figure 5b). Labelled neurons were ovoid or fusiform in shape with their long axis oriented parallel to the dorsal surface of the underlying pars reticulata. In the pars reticulata, the label was evident over both scattered nerve cell bodies and large dendritic processes radiating from the pars compacta (Figure 5c). In the ventral tegmental area, fluo-NT-labelled nerve cell bodies were prominent in amongst cross-sectioned labelled processes (Figure 5d). Finally, in the interfascicular nucleus, the labelling was dense throughout and mainly concentrated over nerve cell bodies.

Binding and internalization of fluo-NT in SN17 hybrid cells.

The distribution of fluo-NT binding to SN17 hybrid cells varied according to both the temperature of labelling and the CLSM optical plane of section. In cells incubated at 4°C, fluorescent labelling was mostly confined to cross-sectional planes acquired at the surface of the cell (Figure 6a). By contrast, in optical sections taken halfway between the slide and the cell surface, only a few "hot spots", 1-2 μm in diameter, were visible along

the border of the cell (Figure 6b).

When fluo-NT-labeled cells were warmed up for 45 min at 37°C, only weak, diffuse fluorescence was visible in sections grazing the cell surface (Figure 6c). By contrast, intense fluorescent labeling was seen to pervade the cytoplasm in optical sections passing through the center of the cell (Figure 6d). This intracellular labelling took the form of small, intensely fluorescent particles, again between 1 and 2μm in diameter. These particles were often concentrated in the immediate surround of the nucleus but were virtually never apparent within the nucleus itself.

DISCUSSION

The present study describes the synthesis of a biologically active fluorescent derivative of the tridecapeptide, neurotensin (fluo-NT) and demonstrates the applicability of this compound to the biochemical, pharmacological and anatomical characterization of high affinity NT receptors in mammalian brain.

Although other examples of conjugation of fluorescent markers to bioactive peptides have been previously reported (10),(11),(13),(39),(40),(41),(42),(43), the present work provides the first description of a direct acylation of the semi-protected peptide ([Glu^1]NT)-resin by FITC, to generate a fluoresceinylated derivative. This approach yielded site-specific labeling of the Nα-amino function of [Glu^1]NT, the protection of the Nα-amino function in position 8 allowing for easier purification of the labeled compound as well as for preservation of the affinity of the ligand for its receptor (44). In our conditions of cleavage and purification, the FTC moiety was stable. The moderate yields of the synthesis/purification procedure on both Nα-FTC-[Glu^1]NT and [Glu^1]NT were mainly due to our high standard of purity (99%) and to the lower recoveries generally obtained with reverse-phase media when small amounts of peptides (=30mg/run) are purified on preparative size columns.

Flow cytometric analysis of fluo-NT binding to SN17 hybrid cells yielded a single narrow peak indicating that virtually all of the clonal cells had bound the fluorescent compound in a relatively homogenous fashion. Mean fluorescence intensity measurements in the presence and absence of non fluorescent NT indicated that in the range of concentrations of fluo-NT used, up to 85% of the total binding was specific, a proportion similar to that obtained when competing on the same cells for the binding of ^{125}I-NT (28). Specific fluo-NT binding was saturable, and reached a plateau at ligand

concentrations comparable to those required for saturation of ^{125}I-NT binding sites (28), suggesting that fluorescent and monoiodinated forms of the ligand bind to high affinity NT receptors with comparable affinities. That this is indeed the case was further supported here by the results of competition experiments on mouse brain homogenates, which showed fluo-NT to compete for ^{125}I-NT binding with the same affinity as previously demonstrated for non radioactive monoiodo-NT (35). Furthermore, the K_I with which fluo-NT was found to compete for ^{125}I-NT binding sites was the same as that of the native peptide, indicating that the addition of an FITC molecule on the N-terminus had not decreased the affinity of the peptide for its receptor. This finding is consistent with the result of previous conjugation experiments which have shown that increasing the steric hindrance in position 6, but not in position 1, decreased the affinity of NT for its specific binding sites (45),(46).

In frozen sections from the rat midbrain tegmentum incubated with 10nM fluo-NT, both conventional epifluorescence and confocal microscopy revealed selective binding of the fluorescent compound throughout the substantia nigra, ventral tegmental area, and interfascicular nucleus. This binding was almost entirely specific as demonstrated by the fact that sections incubated in the presence of an excess of unlabeled ligand showed only weak background fluorescence. At the regional level, the distributional pattern of fluo-NT labeling was comparable to that previously observed by autoradiography in the same region using either tritiated (22),(23),(24) or iodinated NT (25),(47), (Figure 5). However, images obtained in the fluorescent microscope afforded a substantially higher degree of resolution than did autoradiograms. This was particularly true of the images obtained in the confocal microscope, in which the out-of-focus contribution was reduced through the use of a point illumination/point detection technique and the acquisition of thin optical sections (Steps of focus increments of 0.2 μm) (18),(48).

The perikarial labeling detected here in neurons from the substantia nigra, ventral tegmental area and interfascicular nucleus was reminiscent of that previously observed in high resolution autoradiograms of the same region using 1-5 μm-thick Epon-embedded (49) or frozen (47) sections. Furthermore, optical sectioning showed the ligand to be distributed in the form of small intracytoplasmic clusters which had previously escaped autoradiographic detection. The nature of these clusters is still unclear, although some of them may conceivably correspond to Golgi vesicles or to lysosomes, as both of these structure have been found to specifically bind ^{125}I-NT by electron microscopic autoradiography (49),(50). It is interesting in this context that particles of comparable size were detected in SN17 cells incubated at 37°C in vivo, suggesting that some of these particles may correspond to preformed endocytic vesicles.

The distribution of bound fluo-NT was markedly different inside and/or on the surface of dendrites, as attested to by the much more diffuse and floccular aspect of the fluorescent labeling in dendrites than in perikarya. These observations are consistent with those of our earlier electron microscopic study which have shown NT binding sites to be more or less uniformly distributed over the surface of midbrain tegmental dendrites (49) and, in the case of internal sites, to display no preferential association with any given intracytoplasmic organelle (49).

Labeling of SN17 cells with fluo-NT at 4°C yielded a fairly heterogenous labeling pattern characterized by the presence of "hot spots" reminiscent of opioid receptor clusters labeled on neuroblastoma cells with fluoresceinylated met-enkephalin (10),(12) or of glycine receptor "microdomains" labeled by immunohistochemistry and visualized in the confocal microscope on the surface of the goldfish Mauthner cell (51). Whether these hot spots reflect a constitutive heterogeneity in the distribution of NT receptors on the membrane of SN17 cells or are the consequence of ligand-induced receptor aggregation

and sequestration (10),(52) remains to be determined. The warming up of fluo-NT-labeled SN17 cells to 37°C both decreased cell surface reactivity and induced internal labeling, suggesting that the tracer had been internalized into the cells. Experiments carried out with ^{125}I-NT on both SN17 (28) and primary neurons in culture (53) have previously demonstrated the occurence of receptor-mediated endocytosis of NT in neuronal cells. In fact, biochemical studies have indicated that this internalization process was likely to subserve receptor down regulation (54) or even trans-membrane signalling (55). The particulate appearance of internalized fluorescent molecules observed in the present study strongly suggests that the internalization process is carried out through an endosomal compartment, as previously demonstrated in neurons for other, non-transmitter molecules (56).

In conclusion, the present results demonstrate that NT_{1-13} may be fluoresceinylated by substituting Glu for pGlu in position 1. The resulting compound was shown to bind with high affinity to central NT receptors and to be applicable to the cellular localization of high affinity NT binding sites both in live cells and in tissue sections. Fluo-NT was also found to be actively internalized in live neural cells suggesting that it is biologically active. This interpretation is in keeping with the recent demonstration that application of fluo-NT onto basal forebrain cholinergic cells in slices elicits the same type of persistent slow bursting pattern as native NT (57) and implies that our fluorescent conjugate may be used for functional studies of NT receptors in vivo.

Acknowledgments

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FIGURE LEGENDS

Figure 1

Analytical reverse-phase HPLC profile of an admixture of pure [Glu^1]NT and N_-FTC-[Glu^1]NT detected by UV absorbance at 214 nm and 280 nm (inverse profile). The solvent system was composed of 0.01% acqueous TFA, pH 2.9 and CH_3CN-0.01% TFA. A linear gradient of 1% increase of CH_3CN/min was used with 10% CH_3CN as initial condition and a flow rate of 1.5 ml/min. [Glu^1]NT and N_-FTC-[Glu^1]NT were respectively eluted in 25.3% (retention time: 15.3 min) and 38.2% (retention time: 28.2 min) CH_3CN.

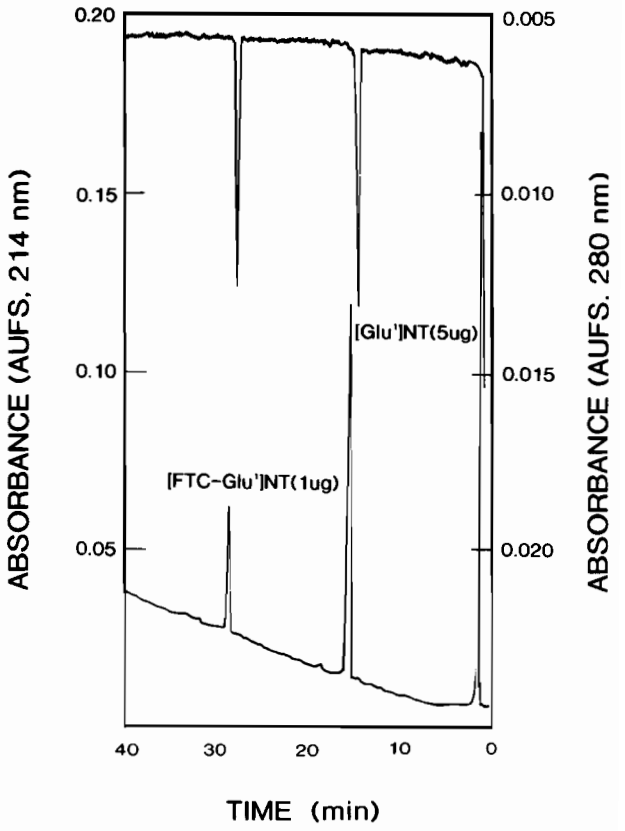
0.20
0.15
0.10
0.05
0.005
0.010
0.015
0.020
ABSORBANCE (AUFS, 214 nm)
ABSORBANCE (AUFS. 280 nm)
[Glu¹]NT(5ug)
[FTC-Glu¹]NT(1ug)
40
30
20
10
0
TIME (min)

Figure 2

Flow cytometric analysis of fluo-NT binding to SN17 hybrid cells. A total number of 5000 live SN17 cells in suspension were incubated at 4°C with 10nM fluo-NT in the presence (non specific binding; left panel) or in the absence (total binding; right panel) of a hundredfold excess of non fluorescent NT. The relative cell number (ordinate) is plotted against the fluorescence intensity of the bound ligand (abscissa, log scale). Each point is the mean of two determinations in a representative experiment.

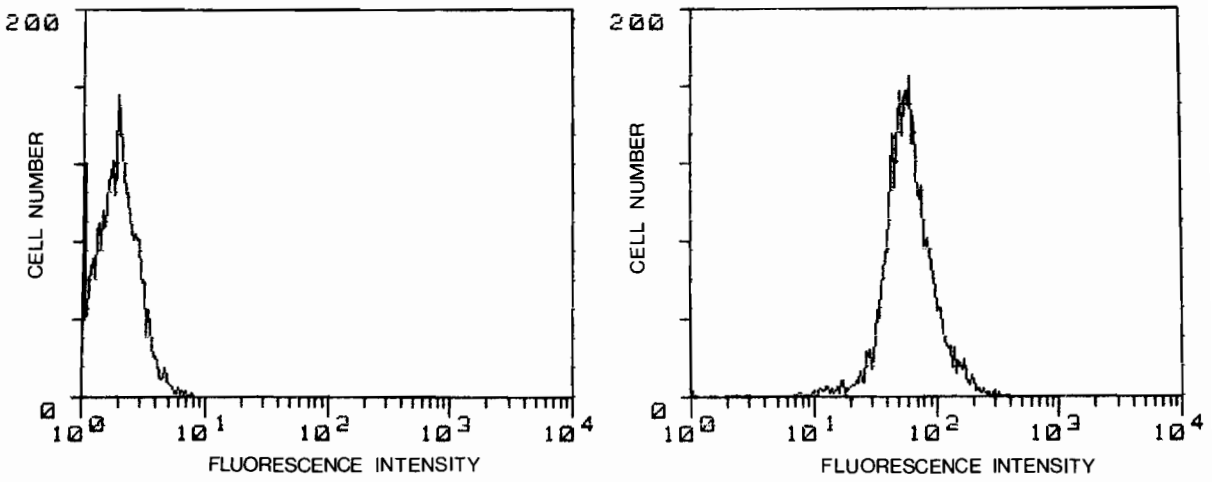
200
CELL NUMBER
0
10⁰
10¹
10²
10³
10⁴
FLUORESCENCE INTENSITY
200
CELL NUMBER
0
10⁰
10¹
10²
10³
10⁴
FLUORESCENCE INTENSITY

Figure 3

Saturation of specific fluo-NT binding (_) to SN17 hybrid cells. Cells were incubated at 4°C with graded concentrations of fluo-NT in the absence (total binding) or the presence (non specific binding) of unlabelled NT and the mean fluorescence intensity (MFI) of the bound ligand measured by flow cytometry. Data are expressed as a function of ligand concentration. Each point is the mean of triplicate determinations in a representative experiment.

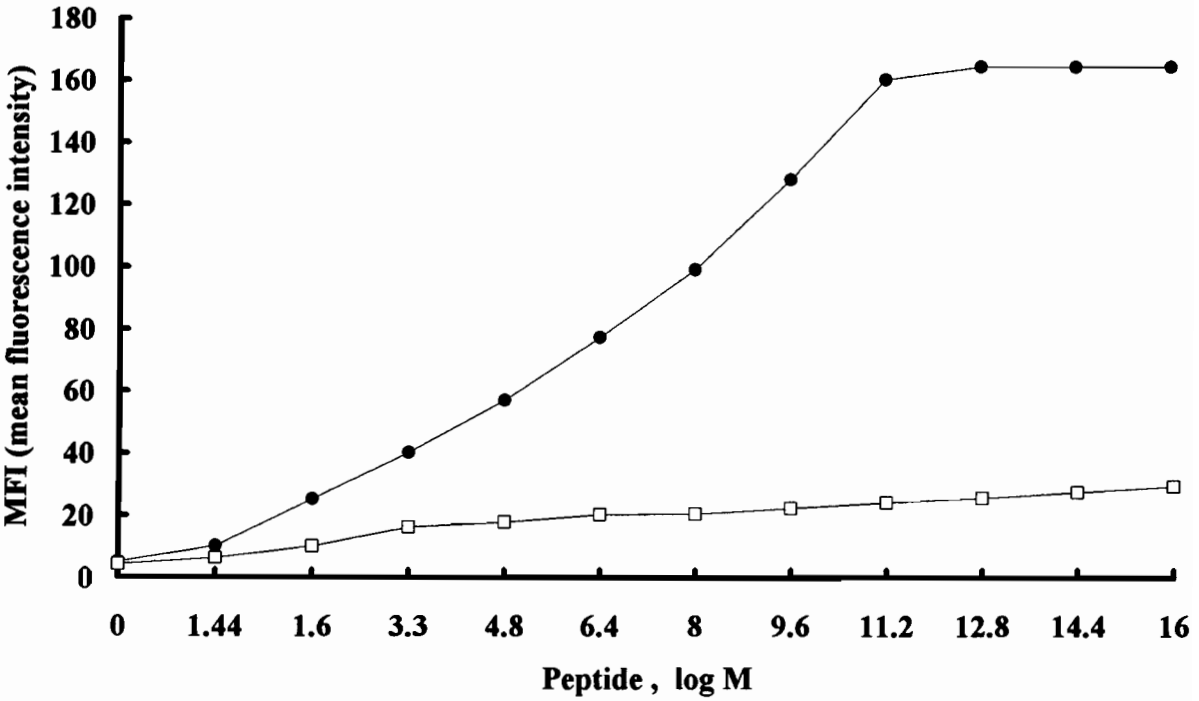
MFI (mean fluorescence intensity)
180
160
140
120
100
80
60
40
20
0
0
1.44
1.6
3.3
4.8
6.4
8
9.6
11.2
12.8
14.4
16
Peptide , log M

Figure 4

Displacement of specific ^{125}I-NT (0.2nM) binding to mouse brain membrane preparations by increasing concentrations of fluo-NT (■) and unlabelled NT (⍥). Each point is the mean of duplicate determinations in a representative experiment.

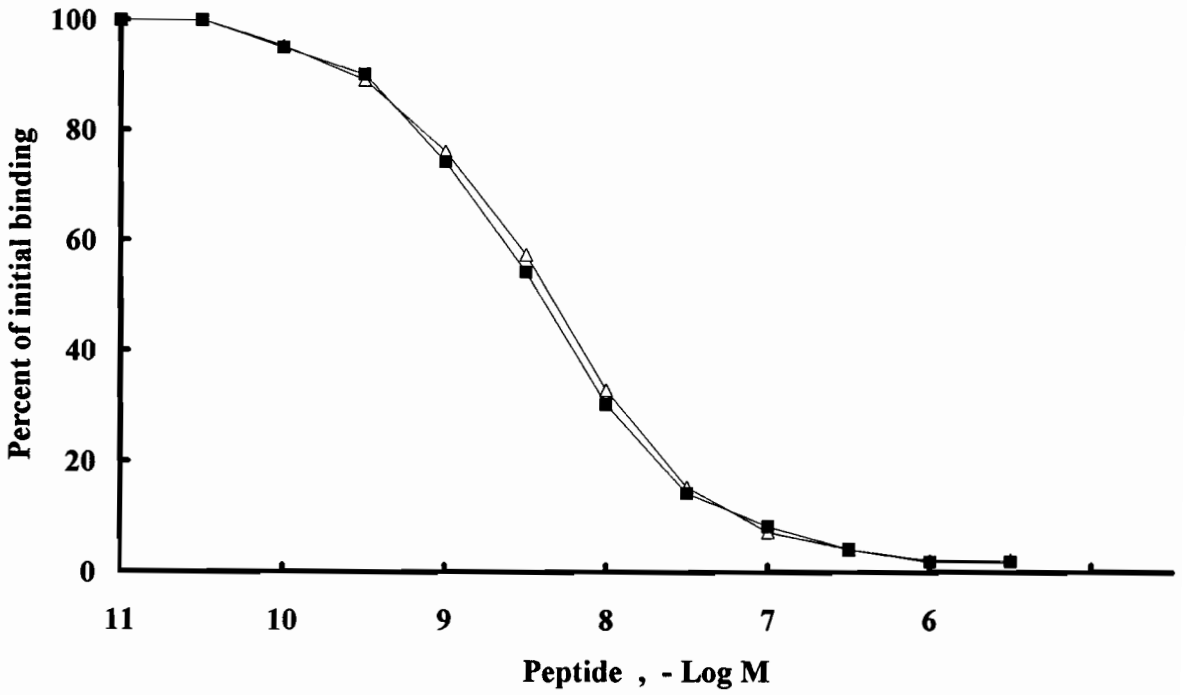
100
80
60
40
20
0
Percent of initial binding
11
10
9
8
7
6
Peptide , - Log M

Figure 5

Confocal laser microscopic imaging of fluo-NT binding to 20 μm-thick sections from the rat midbrain tegmentum. 5μm-thick optical sections. Distribution of bound fluo-NT (b-e) is compared to that of bound ^{125}I-NT as revealed in the same region by film autoradiography (a; excerpted from (25) with permission). b: Substantia nigra, pars lateralis: intensely fluorescent cells and processes form a discrete band of labeling continuous with, and in the axis of, the pars compacta. Scale bar: 40μm. c: Substantia nigra, pars reticulata: fluorescent labelling is mainly evident within large, longitudinally trans-sectioned dendrites originating from neurons in the pars compacta. Scale bar: 40μm. d: Ventral tegmental area: the labelling is apparent over both neuronal perikarya (arrows) and intervening cross-sectioned processes. Scale bar: 20μm e: High magnification of labelled perikarya in the ventral tegmental area. Note the granular distribution of the label in the cytoplasm and the clearcut sparing of the nucleus (N). Scale bar: 20μm. Abbreviations: IF, interfascicular nucleus; IP, interpeduncular nucleus; SNC substantia nigra, pars compacta; SNL, substantia nigra, pars lateralis; SNR substantia nigra, pars reticulata; VTA, ventral tegmental area.

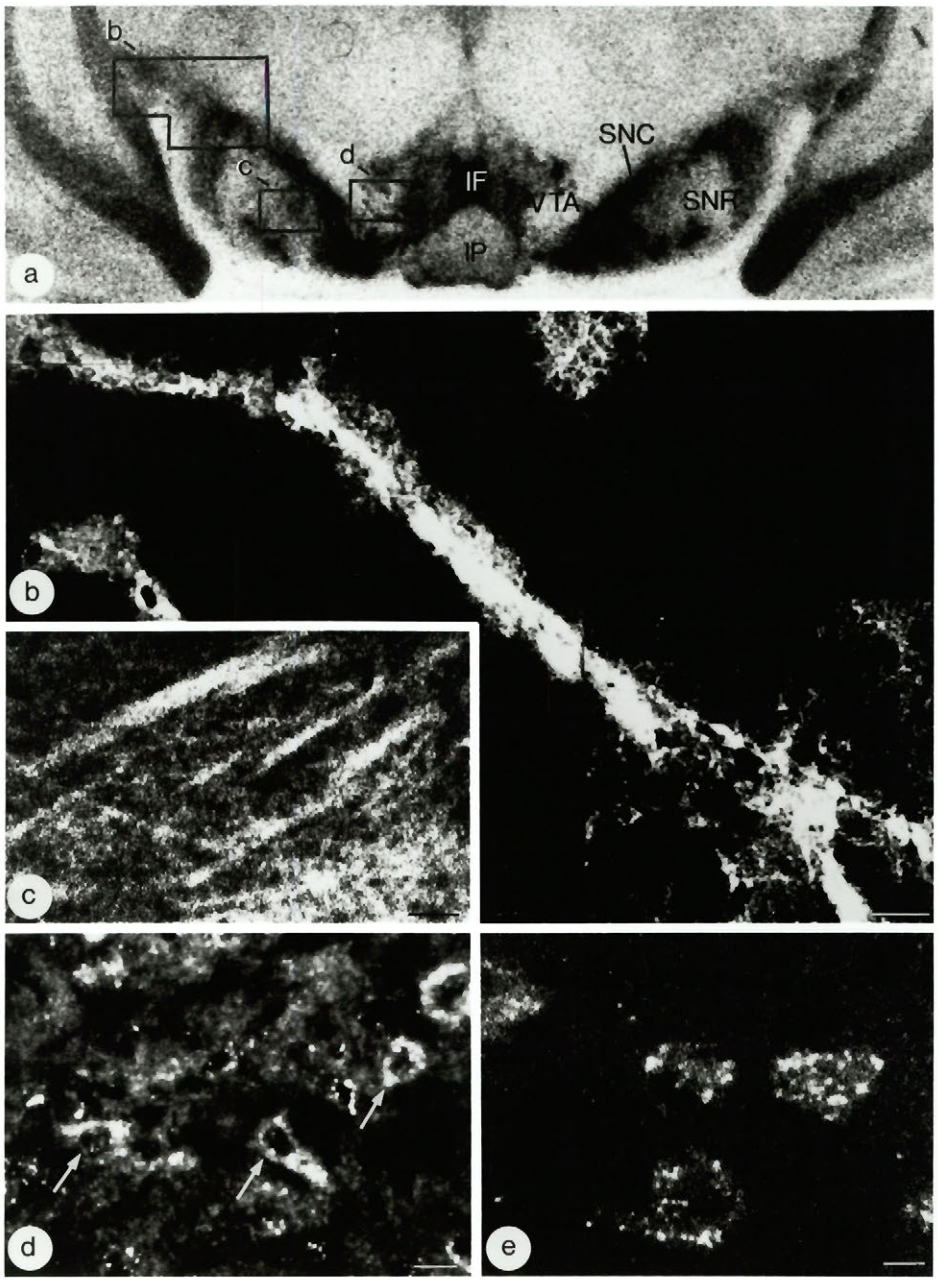
b
c
d
IF
VTA
SNC
SNR
IP
a
b
c
d
e

Figure 6

Confocal fluorescence imaging of fluo-NT labelling of SN17 hybrid cells. Cells were incubated for 60 min at 4°C with 10nM fluo-NT and immediately air dried (a,b) or warmed up to 37°C for a further 45 min prior to examination (c,d). 0.2 µm optical slicing steps. a,c: Optical sections grazing the cell surface (cross-sectional plane no.5, out of 25 consecutive planes taken from the surface of the section inward). b,d: Optical sections passing through the center of the cell. (Section #15/25) Scale bar: 10µm.

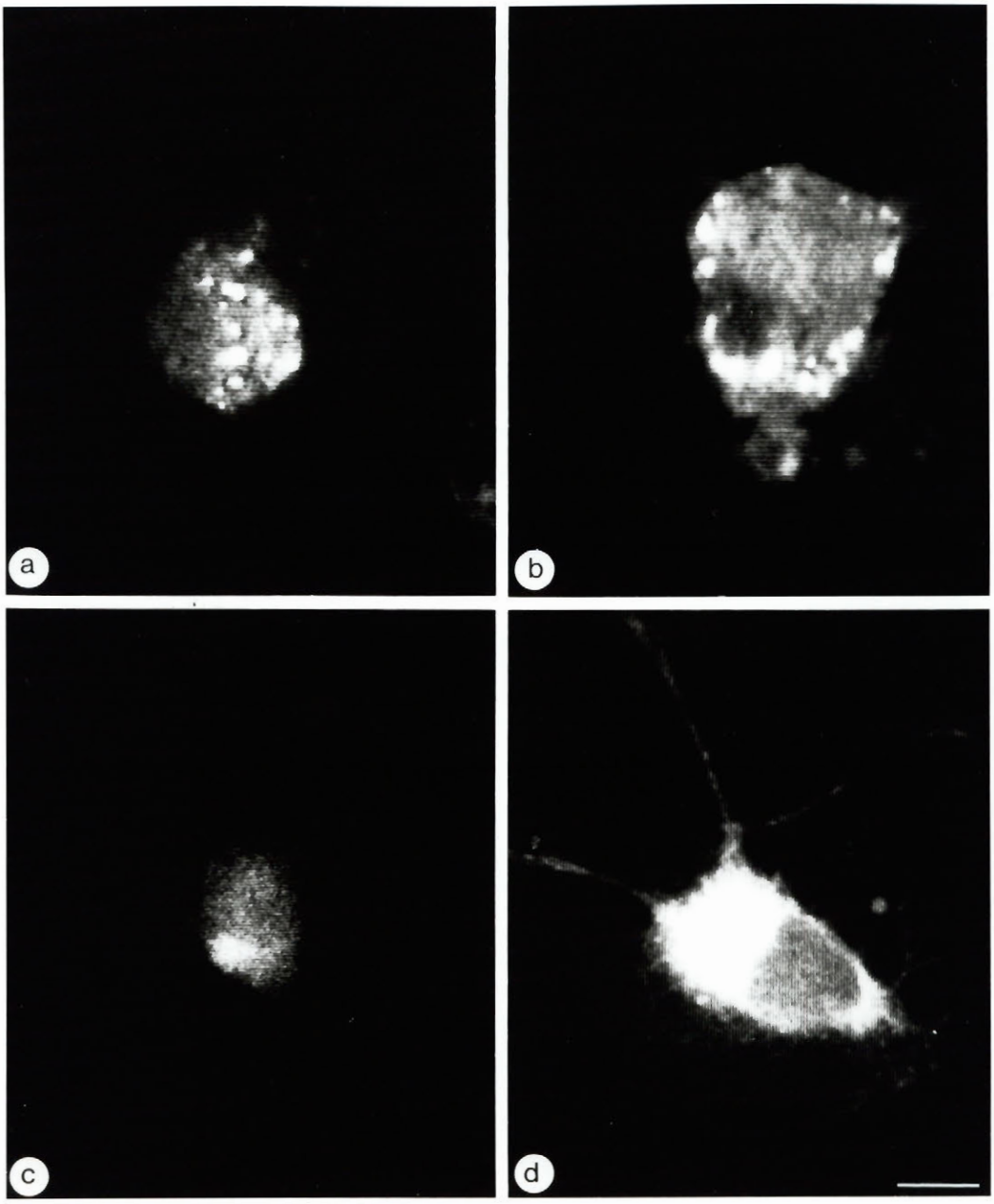
a
b
c
d

TABLE I

Amino acid composition of [Glu^1]NT and N_-FTC-[Glu^1]NT*

	[Glu^1]NT	N_-FTC-[Glu^1]NT
Ans (1) **	0.89	1.06
Glu (2)	1.83	1.30
Arg (2)	1.97	2.41
Pro (2)	2.01	2.40
Tyr (2)	1.83	1.44
Ile (1)	1.05	1.13
Leu (2)	2.14	2.30
Lys (1)	1.16	0.96
Peptide Content (%)	85	82

* Determined after acidic hydrolysis (6N HCl, 110°C, 18h) and PITC derivatization by reverse-phase HPLC (waters PICO-TAG column 0.39 X 15 cm, 38°C) using a solvent system consisting of 140 mM sodium acetate (PH 6.4) and 60% aqueous CH_3CN.

** Numbers in parenthesis refer to theoretical number of amino acids included in each peptide.

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Connecting text #1

We have synthezised and characterized a marker for NT-R which labels cell receptors in fresh frozen sections and cell culture as identified by confocal laser microscopy. We then utilized this tool to better understand NT-R function in cholinergic neurons previously shown to express NT-R.

CHAPTER 3

SOMATODENDRITIC INTERNALIZATION AND PERINUCLEAR TARGETING OF NEUROTENSIN IN THE MAMMALIAN BRAIN.

Marie-Pierre Faure [1*], Angel Alonso [1], Dominique Nouel [1], Georges Gaudriault [2], Michael Dennis [3], Jean-Pierre Vincent [2] and Alain Beaudet [1].

[1] Montreal Neurological Institute, 3801 University Street, Montreal, Quebec, H3A 2B4, Canada. [2] Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, UPR 0411, Université de Nice Sophia-Antipolis, 660 Route des Lucioles, Sophia-Antipolis, 06560 Valbonne, France. [3] BioSignal Inc., 1744 William Street, Montreal, Quebec, H3J 1R4, Canada.

30 text pages, 4 plates, 1 table

Key words: neurotensin; receptors; basal forebrain, confocal microscopy

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ABSTRACT

Polypeptide hormones and growth factors have long been known to internalize into peripheral target cells as a result of their interaction with cell surface receptors. Studies in culture have suggested that certain neuropeptides might undergo a similar type of translocation in neurons. To investigate this possibility in adult mammalian brain, we have examined by confocal-laser microscopy the events that follow the binding of fluorescein-tagged derivatives of the tridecapeptide neurotensin to basal forebrain cholinergic cells. Our results demonstrate a selective time- and temperature-dependent internalization of fluo-neurotensin in these cells. This internalization is receptor-mediated, proceeds from the entire somatodendritic membrane of the cells and utilizes endosome-like organelles which are mobilized from dendrites to perikarya and from the periphery of the cell to its perinuclear region. Parallel studies carried out on Sf9 insect cells expressing the rat neurotensin receptor from a recombinant baculovirus indicated that the internalization process involves receptor-ligand complexes and not merely the fluorescent peptide itself. These data suggest that receptor internalization plays a role in neuropeptide signalling in the brain and that it can be harnessed for selective identification of neuropeptide target cells.

INTRODUCTION

A variety of signalling molecules, mainly comprised of peptide hormones and growth factors, have been shown to rapidly enter their target cells after interacting with specific cell surface receptors (Pastan and Willingham, 1981; Posner et al., 1981; Goldstein et al., 1985; Smythe and Warren, 1991). This process, referred to as receptor-mediated internalization, is believed to involve local clustering of the receptors followed by their endocytosis via clathrin-coated pits (Pastan and Willingham, 1981; Goldstein et al., 1985; Keen, 1990; Sorkin and Carpenter, 1993). Although mainly documented for large polypeptides acting through receptor tyrosine kinases (Carpenter and Cohen, 1976; Posner et al., 1981; Knutson, 1991; Wiley et al., 1991; Sorkin and Waters, 1993), receptor-mediated internalization also has been reported for a number of smaller peptides acting through G-protein-coupled receptors. For instance, biochemical and/or histochemical studies have provided evidence for receptor-induced internalization of somatostatin (Morel et al., 1986) as well as of gonadotropin (Hazum et al., 1980; Naor et al., 1981; Pelletier et al., 1982; Duello et al., 1983; Wynn et al., 1986; Morel et al., 1987), corticotropin (Leroux and Pelletier, 1984) and thyrotropin (Morel et al., 1985) releasing hormones in cells of the anterior pituitary. There have also been reports on the internalization of cholecystokinin (Williams et al., 1982), vasoactive intestinal peptide (VIP; Svoboda et al., 1988; Anteunis et al., 1989) and somatostatin (Viguerie et al., 1987) in pancreatic acinar cells, of VIP in intestinal epithelial cells (Izzo et al., 1991), of vasopressin in kidney and smooth muscle cells (Lutz et al., 1990) and of angiotensin II (Husain et al., 1987) in adrenal glomerulosa cells.

Recent work on the tridecapeptide, neurotensin (NT) has suggested that this process of receptor-mediated internalization may be operational not only in peripheral

organs, but also in nervous tissue. Thus, autoradiographic and/or biochemical studies have shown radioactive NT to be rapidly taken up by mouse and rat neurons in culture in a temperature- and receptor-dependent fashion (Mazella et al., 1991; Vanisberg et al., 1991; Beaudet et al., 1994). This internalization process was shown to initially promote the rapid appearance of a new pool of NT binding sites on the cell membrane (Chabry et al., 1993) and to later result in a down-regulation of cell surface receptors (Vanisberg et al., 1991). It has also been implicated in the initiation of the retrograde axonal transport of ^{125}I-NT observed in nigro-striatal neurons following intra-striatal injections of this radioligand (Castel et al., 1990, 1992; Beaudet et al., 1994). However, there is still no information on the extent and selectivity of NT internalization in mammalian brain nor on the mechanisms that subserve it. It is unclear, in particular, whether this phenomenon: (i) is linked to receptor activation; (ii) involves the ligand alone or receptor-ligand complexes; (iii) proceeds from the entire neuronal surface or is restricted to specialized areas of the cells; and (iv) is carried out through classical endocytic pathways.

To clarify some of these issues, we have examined the binding and internalization of novel fluorescein-tagged derivatives of NT in slices of the rodent basal forebrain. This region was selected because it had previously been shown to harbour high concentrations of NT receptors (Moyse et al., 1987) which are selectively associated with the perikarya and dendrites of acetylcholinesterase containing (i.e. of presumptive cholinergic) neurons (Szigethy and Beaudet, 1987; Szigethy et al., 1989). To further document the cellular mechanisms underlying NT internalization and determine whether this process was mediated through the cloned NT receptor (Tanaka et al., 1990), parallel studies were carried out on Sf9 insect cells expressing the rat NT receptor from a recombinant baculovirus. This model system, which has previously been used to study the biochemical properties of other types of G protein-coupled receptors (Mouillac et al., 1992), has the

advantage of generating considerably higher levels of receptors than mammalian cells while maintaining their normal pharmacological properties (Wong et al., 1990; Mouillac et al., 1992).

MATERIALS AND METHODS

Fluorescent Ligands

Three different fluorescent-tagged NT derivatives were used in the present experiments. The first (Nα--fluoresceinyl thiocarbamyl (FTC)-[Glu^1]NT) was selectively labeled on the terminal α-amine function of [Glu^1] NT using a solid phase methodology and purified by reverse phase HPLC as described (Faure et al., 1994). The second (Nα-fluoresceinyl-NT (2-13)) was obtained by incorporating a fluoresceinyl group on the terminal α-amine function of NT (2-13). This derivative was prepared by reacting the N-hydroxysuccinimide ester of fluorescein with NT (2-13) at pH 6.5 (Gaudriault and Vincent, 1992) and purified by reverse phase HPLC. Both of these compounds were found to inhibit specific ^{125}I-NT binding to mouse brain membrane preparations with apparent affinities (Ki) virtually identical to those of the native peptide (Faure et al., 1994; Gaudriault and Vincent, unpublished). They also yielded identical confocal microscopic results and were hence used interchangeably. Both are referred to in the text under the generic term fluo-NT.

The third compound (fluo-azido-nitro NT) was a photoreactive derivative of Nα-fluoresceinyl-NT (2-13). In addition to the fluorophore incorporated on its N-terminal end, this analogue was also substituted on the α-amine function of Lys6 by an azido-nitro group. The fluorescent photoreactive analgue was synthesized by reacting Nα-fluoresceinyl-NT (2-13) with sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, Pierce) at pH 8.5 (Gaudriault and Vincent, 1992) and purified by reverse phase HPLC. The advantage of this compound is that it may be covalently cross-linked to receptor proteins by photoactivation at 320-350 nm, a condition which limits damage to biomolecules and cells by irradiation (Ballmer-Hofer et al., 1982).

However, because of the presence of a reactive group on the side-chain of Lys6, the affinity of the photoreactive analogue for the NT receptor is approximately 5 times lower than that of NT (Mazella et al., 1985).

Labeling of NT Receptors in Brain Slices

Adult male Sprague-Dawley rats (n=10) were killed by decapitation. The brain was rapidly removed and immersed in a cold oxygenated (95% O_2, 5% CO_2) Ringer solution containing 130mM NaCl, 20mM $NaHCO_3$, 1.25mM KH_2PO_4, 1.3mM $MgSO_4$, 5mM KCl, 10mM glucose and 2.4mM $CaCl_2$. Blocs of basal forebrain were sliced at 350µm thickness on a vibratome. The slices were equilibrated for 45 min in oxygenated Ringer at room temperature, superfused for 3 min with 20nM fluo-NT at 37°C and rinsed with oxygenated Ringer for 5, 10, 15, 30, 45 and 60 additional min at 37°C. To control for non-specific labeling, additional slices were incubated in the presence of 2µM non-fluorescent NT or with 20nM free fluorescein in lieu of fluo-NT. To determine whether the labeling was due to endocytosis, fluo-NT incubations were carried out at 4°C or at 37°C after pre-loading the superfusion buffer for 10 min with 10µM phenylarsine oxide. After rinsing, all slices were fixed for 30 min at room temperature with 4% paraformaldehyde in 0.1M phosphate buffer, placed overnight in 30% sucrose in the same buffer, snap frozen on the stage of a freezing microtome and resectioned at 45µM thickness for confocal microscopic examination.

For combined detection of internalized fluo-NT and choline acetyltransferase (ChAT) immunoreactivity, fluo-NT-labeled frozen sections were washed in 3 consecutive baths of 0.1M Tris buffered-saline (TBS), incubated 30 min in 3% normal rabbit serum in TBS and then overnight with a 1/400 dilution of ChAT monoclonal antibody (Incstar, Stillwater, Mn) in TBS containing 1% rabbit serum. After several washes (3 x 5 min) in

TBS, the primary antibody was revealed with a 1/100 dilution of biotinylated goat anti-rat antibody (60 min at room temp) followed by streptavidin-Texas red (1/100; 30 min at room temp). Sections were then washed 3 x 5 min in TBS, mounted with Aquamount and analyzed by confocal microscopy.

Baculovirus Construction and NTR Expression in Sf9 Cells

A recombinant baculovirus was constructed which encoded the rat NTR cDNA (kindly provided by S. Nakanishi, Kyoto University) using procedures described elsewhere (Mouillac et al., 1992; M. Dennis, manuscript in preparation). Briefly, the cDNA was subcloned into the baculovirus transfer plasmid pJVETLZ/Nhel and recombinant viruses isolated by plaque purification following co-transfection of plasmid and wild-type AcNPV viral DNA into Sf9 cells. For expression, cultures of Sf9 cells were grown in spinner flasks in Grace's medium containing 10% fetal bovine serum and infected with the recombinant virus at a multiplicity of infection of 2.

Expression of the NTR in Sf9 cells was verified by radioligand binding to membrane preparations. Sf9 cells infected with the NTR virus were harvested at 48 h post-infection and membranes prepared, as previously described (Mouillac et al., 1992). The membranes (10μg of protein) were incubated for 20 min at 25°C in 50 mM Tris-HCl buffer (pH 7.5) containing various concentrations of α-^{125}I Bolton Hunter-NT (2-13), 1 mg/ml (Gaudriault and Vincent, 1992). The incubation was stopped by addition of 2 ml ice-cold buffer and rapid filtration under reduced pressure through cellulose acetate filters (pore size, 0.2nm; Sartorius). Filters were washed twice with 2 ml of ice-cold buffer. Radioactivity retained on filters was counted with a gamma counter at a counting efficiency of 80%. Non-specific binding was measured in the presence of an excess (1μM) of unlabeled NT and subtracted from total binding to obtain specific binding.

Fluo-NT-Labeling of Infected Sf9 Cells

Fourty eight hours after baculovirus infection, Sf9 cells were rinsed twice in 50mM Earle's buffer, pH 7.4, containing 140mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 3.6mM $MgCl_2$, 0.1% bovine serum albumin and 0.01% glucose, equilibrated for 10 min in the same buffer and labeled with either fluo-NT or fluo-azido-nitro NT.

For fluo-NT labeling, cells (5 x 10^4/ml; 0.5ml/assay) were incubated with 10nM fluo-NT in the same buffer for 60 min at either -5°C or 21°C and in the presence or absence of 10μM of the endocytosis inhibitor, phenylarsine oxide. At the end of the incubation, the cells were washed 4 x 1 min in cold binding buffer, deposited on glass slides, dried under a cool stream of air and examined in the confocal microscope under oil immersion. To control for non-specific binding, the experiments were carried out in the presence of 1μM non-fluorescent NT or on Sf9 cells infected with a baculovirus encoding the human ß-adrenergic receptor (Mouillac et al., 1992).

For fluo-azido-nitro NT labeling, Sf9 cells were incubated in the dark for 30 min at -5°C with 20nM of fluo-azido-nitro NT in the same buffer as above with and without a hundred fold excess of native NT for determination of non-specific binding. At the end of the incubation, the cells were subjected or not to 3 consecutive photographic flashes, washed 4 x 1 min in cold binding buffer and either fixed in 4% paraformaldehyde in 0.1M PO_4 buffer for 20 min and dehydrated in graded ethanols for confocal microscopic viewing or warmed up to 21°C for a further 45 min prior to being fixed and dehydrated as above.

Confocal Microscopy

Basal forebrain sections and labeled Sf9 cells were both examined under a Leica confocal laser scanning microscope (CLSM) configured with a Leica Diaplan inverted microscope equiped with an argon ion laser (488 nm) with an output power of 2-50mV

and a VME bus MC 68020/68881 computer system coupled to an optical disc for image storage (Leica, St. Laurent, Canada). All image generating and processing operations were performed with the Leica CLSM software package. Micrographs were taken from the image monitor using a Focus Imagecorder (Foster City, California).

Images of the basal forebrain were acquired according to two different modes: (1) serial 0.24µm-thick optical sections averaged over 4 scans/frame and reconstructed (extended focus) over a depth of 3µm; (2) individual 10µm-thick optical sections averaged over 128 scans/frame for high resolution imaging. Images of Sf9 cells were acquired as single 0.42µm-thick optical sections and averaged over 32 scans/frame. For all types of acquisitions, the gain and black levels were set manually to optimize the dynamic range of the image while ensuring that no region was completely suppressed (intensity = 0) or completely saturated (intensity = 255).

Double fluorescence images were acquired in two passes, fluorescein first, Texas red second, to avoid bleeding from one channel into the other. Fluorescent polystyrene beads (diameter 10µm; Becton Dickinson) were used to verify that the two emission filters were properly aligned.

Fluorescence intensity measurements were performed on reconstructed serial images of the basal forebrain (acquisition mode #1) and expressed as grey levels per unit area on a 0-255 scale. Values were averaged for 12-14 readings from at least 14 different sections. Morphometric measurements of the diameter, number and distance from the nuclear center of intracytoplasmic fluorescent particles were performed on individually scanned 10µm-thick sections (acquisition mode #2). Results correspond to the mean ± SEM of 4 sections. Statistical analyses were performed using one way analysis of variance (one-way ANOVA), followed by a regression curve analysis. The comparison of the slope from the regression curve was done using a Student's t-test.

RESULTS

Studies on Brain Slices

Five min after a 5 min superfusion of rat basal forebrain slices with 20nM fluo-NT at 37°C, intense and pervasive fluorescent labeling was detected by confocal microscopy in a selective subset of neurons distributed throughout the medial septal nucleus, the diagonal band of Broca and the substantia innominata. Although most prominent over neuronal perikarya, the labeling was also apparent over multiple cross-sectioned neuronal processes throughout the neuropil (Fig. 1a). Quantification of the fluorescent signal revealed a 1.5-fold difference in intensity between perikaryal and neuropil labeling at this time (Table 1). By contrast, sections incubated with a thousand-fold excess of native NT, or with free fluorescein in lieu of fluo-NT, showed no significant fluorescent signal over background noise, indicating that fluo-NT labeling was dependent upon specific binding of fluo-NT to NT receptors. Slices exposed to fluo-NT at 4°C, or at 37°C in the presence of the endocytosis inhibitor, phenylarsine oxide were also devoid of significant fluorescent signal indicating that the observed labeling resulted from a temperature- dependent endocytic process. In conformity with these data, serial optical sectioning of fluo-NT-labeled cell bodies revealed that most, if not all, of the observed fluorescent signal was intracellular. High resolution scanning of single 10μm-thick optical sections passing through the core of the cells further indicated that the internalized fluorescence was confined to small, spherical particles distributed throughout the cytoplasm (Fig. 1c).

Between 5 and 30 min after application of the fluorescent ligand, the labeling became progressively more intense over neuronal perikarya and proportionally less intense over neuronal processes (Table 1). By 60 min, the fluorescent signal had increased by 19% over nerve cell bodies and had dropped to background levels in the surrounding

neuropil (Fig. 1b; Table 1). Throughout this time period, the label remained in the form of small, intensely fluorescent particles (Fig. 1d). However, the mean diameter of these particles increased linearly with time ($F(1,20)=11.4, p<0.01$; Fig. 2A). In addition, the number of particles per unit of labeled cell cross-sectional area decreased linearly with time ($F(1,19)=13.38$, $p<0.01$; Fig. 2B). These two events were significantly inversely correlated ($r=-0.98; p<0.001$). Finally, when the mean distance between each perikaryal fluorescent particle and the center of the nucleus was plotted as a function of time, a statistically significant overall translation of the particles from the periphery to the center of the cell was apparent ($F(1,11)=4.86, p<0.05$; Fig. 2C). This movement was such that by 60 min, the bulk of fluorescent particles were confined to the perinuclear zone (Fig. 1d).

To demonstrate that the observed cellular labeling was selective for cholinergic neurons, i.e. for neurons known to be selectively endowed with NT receptors in this region of the brain, slices exposed to fluo-NT at 37°C were washed for 45 min and double-labeled by immunofluorescence using a specific antibody against the acetylcholine biosynthetic enzyme, choline acetyltransferase (ChAT). Confocal microscopic examination of this dually stained material revealed that the large majority of the cells in which fluo-NT had internalized (88.9±2.0%) also stained positively for ChAT (Fig. 1e,f).

Studies on Sf9 Cells

To further characterize the mechanisms underlying the internalization of fluo-NT molecules, experiments were conducted with Sf9 insect cells infected with a baculovirus encoding the cloned NT receptor. Membranes prepared from infected cells at 48 h post-infection bound ^{125}I-NT with a maximal binding capacity (Bmax) of 12.4 pmol/mg protein and an apparent dissociation constant (Kd) of 0.38 nM. Scatchard analysis of the data (not shown) indicated the presence of a single class of NT binding sites.

In keeping with our observations in brain slices, incubation of whole Sf9 cells with 10nM fluo-NT 24-36 h following infection with the NTR baculovirus resulted in a time-dependent internalization of the fluorescent probe (Fig. 3a). As in neurons, this internalization process was no longer observed at low temperature or in the presence of the endocytosis inhibitor, phenylarsine oxide (Fig. 3b). Neither binding nor internalization of fluo-NT were observed in the presence of an excess of non-fluorescent NT or in control Sf9 cells infected with a baculovirus encoding the human ß-adrenergic in lieu of the rat NT receptor.

To determine whether the internalization process involved the ligand alone or ligand-receptor complexes, Sf9 cells were incubated with fluo-azido-nitro NT, a photoaffinity derivative of fluo-NT, in lieu of fluo-NT. In a first step, cell surface receptors were labeled at -5°C, a temperature at which no ligand internalization had been observed using fluo-NT. The cells were then subjected to a photographic flash and washed thoroughly to dissociate non-cross-linked ligand molecules from their receptors. As can be seen in Fig. 4, the labeling achieved under these conditions was confined to the cell surface (Fig. 4a) and was not detectable in cells that had not been exposed to a flash of light (Fig. 4b). Both irradiated and control (non- irradiated) cells were then warmed to room temperature for a further 45 min. At this time, the pattern of labeling in irradiated cells was undistinguishable from that observed with fluo-NT, in that it was entirely intracellular and in the form of small, intensely fluorescent endosome-like particles (Fig. 4c). Here again, cells that had not been subjected to photoirradiation remained label free (Fig. 4d).

DISCUSSION

The present results provide the first demonstration of receptor-induced internalization of a neuropeptide in live brain slices. Evidence that this internalization represents a true receptor-mediated event and not merely a non-specific endocytic process includes: (i) the fact that the labeling was no longer apparent when the incubation was carried out in the presence of an excess of non-fluorescent NT; (ii) the absence of internalization of free fluorescein; and (iii) the selectivity of the internalization process for cholinergic cells which earlier autoradiographic studies had shown to be selectively endowed with high affinity NT binding sites in the basal forebrain (Szigethy and Beaudet, 1987; Szigethy et al., 1989). That the internalization process was indeed selective for cholinergic cells was demonstrated here by the results of our double labeling experiments which showed close to 90% of fluo-NT-labeled cells to stain positively for choline acetyltransferase. In keeping with these observations, electrophysiological studies from our laboratory (Alonso et al., 1994) have demonstrated that bath application of fluo-NT onto slices of the guinea pig basal forebrain induced membrane depolarization and the emergence of a slow complex rhythmic bursting pattern in neurons exhibiting electrophysiological properties characteristic of cholinergic cells (Khateb et al., 1992). Fluo-NT clearly acted as an agonist since native NT induced equivalent responses when applied to the same neurons (Alonso et al., 1994). The internalization process documented in the present study is therefore likely to be triggered by receptor activation, although the sequence of events that link these two phenomena remains to be established.

Neurons expressing the NT receptor cloned by Tanaka et al. (1990) have been visualized in the basal forebrain of the rat using *in situ* hybridization histochemistry (Elde et al., 1990; Sato et al., 1992; Nicot et al., 1994) suggesting that this isoform might be

responsible for the internalization of fluo-NT observed in the present study. Our binding experiments on membranes of Sf9 cells infected with a baculovirus encoding this molecular form of the receptor indicated that ^{125}I-NT bound to the transfected receptor with a Kd value in good agreement with that reported for the NT receptor in rat brain. Confocal microscopic studies of these cells using fluo-NT indicated that these cell surface receptors could indeed mediate internalization of the peptide, in conformity with recent biochemical data from mammalian cell lines transfected with the same clone (Chabry et al., 1994; Hermans et al., 1994b).

The fact that the internalization of fluo-NT could be blocked in both basal forebrain cholinergic and Sf9 infected cells by either lowering the temperature or preincubating the cells with the endocytosis inhibitor phenylarsine oxyde indicated that the internalization process was endocytic in nature. Indeed, the pattern of punctate intracellular fluorescence observed in our material was reminiscent of that described following receptor-mediated endocytosis of fluorescent peptide ligands in other cell types (Hazum et al., 1980; Naor et al., 1981; Lutz et al., 1990). Experiments in Sf9 cells showed this pattern to remain essentially unchanged when the ligand was covalently cross-linked to the receptor prior to internalization demonstrating that this endocytic process involved receptor-ligand complexes and not merely the fluorescent probe.

In brain slices, internalization of fluo-NT molecules was found to proceed from the entire somatodendritic arbor of basal forebrain cholinergic cells, in conformity with our earlier electron microscopic demonstration of a diffuse distribution of NT receptors on the membrane of these cells (Szigethy et al., 1990). The internalization process was rapidly followed by a concomitant decrease in neuropil labeling and increase in perikaryal fluorescence which can best be explained by a migration of internalized receptor-ligand complexes from dendrites to nerve cell bodies. Studies on embryonic neurons in culture

have previously documented the existence of a similar dendritic transport for constitutively internalized molecules (e.g., transferrin, horseradish peroxidase; Parton et al., 1992) but the present results provide, to our knowledge, the first experimental evidence for a dendritic transport of internalized neuropeptide molecules within nerve cells.

The time-dependent reduction in number and inversely correlated increase in size of intracellular fluorescent granules observed in the course of their migration from the periphery to the perinuclear region of the cells is congruent with the evolution of endosomes into multivesicular bodies and ultimately into lysosomes reported in other cell types (Helenius et al., 1983; Hopkins et al., 1990; Van Deurs et al., 1993). This process usually results in a dissociation of receptor-ligand complexes through rapid acidification of the endosomal compartment and ultimately to differential routing of peptide and receptor, the former to the lysosomal compartment for degradation and the latter to the plasma membrane for recycling (Helenius et al., 1983; Schmid et al., 1988). Had these mechanisms been at place here, however, acidification of the endosomal compartment should have quenched the fluorescence of our FITC-tagged ligand (Lutz et al., 1990) which it did not. Furthermore, recent biochemical studies on cerebral neurons in culture have demonstrated that the density of cell surface NT receptors was unaffected by the carboxylic ionophore monensin, a compound known to disrupt intracellular traffic and the recycling of many receptors, from which it was concluded that NT receptors were not recycled (Chabry et al., 1993). Finally, studies of *in vivo* retrograde transport of ^{125}I-NT in nigro-striatal neurons have shown intracellular ^{125}I-NT molecules to be remarkably resistant to metabolic degradation for up to 4 h after ^{125}I-NT injection (Castel et al., 1991), a finding difficult to reconcile with the exclusive routing of internalized NT towards lysosomes. Thus, although additional studies using specific compartment markers are clearly required for further identification of fluo-NT's sequestration compartments,

available data suggest that these may differ significantly from those of classical endocytic pathways.

The physiological significance of the internalization and somatopetal transport of NT demonstrated here in basal forebrain cholinergic cells is still largely conjectural. One obvious function is the clearance of bound ligand molecules. This interpretation is supported by the recent demonstration of immunoreactive endopeptidase 24.16, a metallopeptidase involved in the functional inactivation of NT, within putative NT target cells in the rat CNS (Woulfe et al., 1992). Internalization of receptor-ligand complexes has also been linked to "cellular" (as opposed to molecular) desensitization (Laduron, 1994). Indeed, internalization of NT in rat embryonic neurons in culture has been shown to result in a rapid disappearance of NT receptors from the cell surface (Vanisberg et al., 1991). However, internalization should not be viewed as the only mechanism through which NT receptor desensitization may occur since while rapid densensitization of agonist-induced calcium mobilization was observed in transfected PC 12 cells expressing the rat NT receptor (Hermans et al., 1994a), virtually no desensitization could be detected in CHO cells transfected with the same receptor (Hermans, 1994b).

Although the present results are clearly suggestive of an intracellular routing of internalized receptor-ligand complexes through intracellular degradation pathways, and thereby of a role of internalization in NT receptor down-regulation, the possibility that the internalization process be also involved in the process of long-distance signalling to elicit long term effects of the ligand in target cells, such as proposed for cytokines, growth factors and neuropeptides (see Laduron, 1992 for a review), cannot be excluded. In fact, such a mechanism has been invoked to account for the increase in the expression of tyrosine hydroxylase observed in nigrostriatal dopaminergic neurons following intrastriatal injection of NT (Burgevin et al., 1992). The aggregation of internalized ligand molecules

in the perinuclear zone observed here in the long term, together with the preservation of NT in unmetabolized form within intracellular sequestration compartments following its intracerebral injection (Castel et al., 1991) is consistent with the hypothesis of an intracellular action of the internalized ligand, receptor or fragment thereof (see Laduron, 1994).

A variety of neuropeptides have been shown to internalize in peripheral target cells and are therefore likely to internalize within CNS neurons in light of the present results. The possibility that the process described here for NT may thus represent a common consequence of neuropeptide binding to central receptors should therefore be taken into consideration for the interpretation of *in vivo* pharmacological, including positron emission tomographic data. In fact, the internalization process may even be used as a tool to selectively identify, as demonstrated in the present study, neuropeptide target neurons in live brain.

FIGURE LEGENDS

Figure 1.

a-d: Confocal microscopic imaging of fluo-NT labeling in rat basal forebrain slices. Sections scanned after 5 (a,c) and 60 (b,d) min of fluo-NT washout. Images in a and b are reconstructed from a stack of 12 serial 0.24μm-thick optical sections scanned at low resolution (4 scans/frame). At 5 min (a), labeling in the diagonal band of Broca is evident over both perikarya (arrows) and neuropil. Arrowheads mark the base of the brain. At 60 min (b), nerve cell bodies in the same region stand out against a markedly reduced neuropil labeling. Images in c and d are 10μm-thick single optical sections scanned at high resolution (128 scans/frame). At 5 min (c) fluo-NT is seen to be contained in small, endosome-like particles distributed throughout the cytoplasm of a single NT-receptive cell. At 60 min (d), labeled cells (arrows) exhibit a lesser number of larger fluorescent particles. e,f: Basal forebrain neuron dually labeled for fluo-NT (e) and ChAT (f). Images were acquired as in a and b, but using two different channels to avoid an overlap of fluorophore emission spectra. Note the presence in e of endosome-like particles that are not visible in the ChAT-immunoreactive cell. N: nucleus. Scale bars: a-d: 10μm; e,f: 5μm.

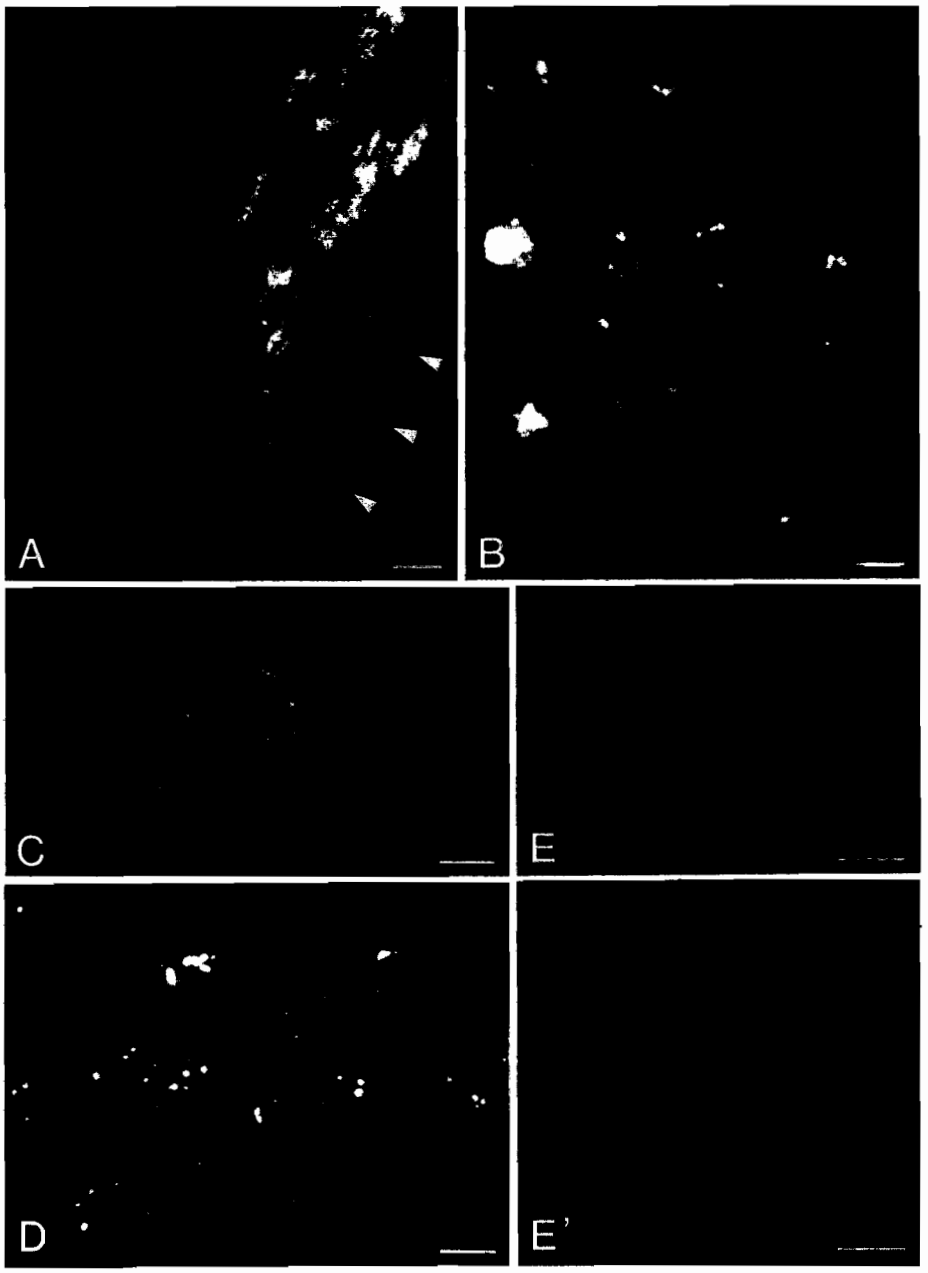
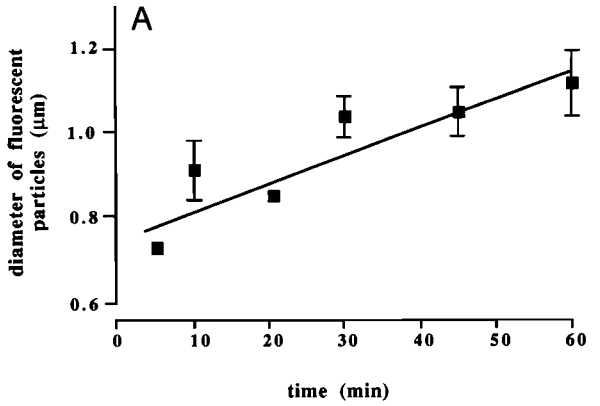
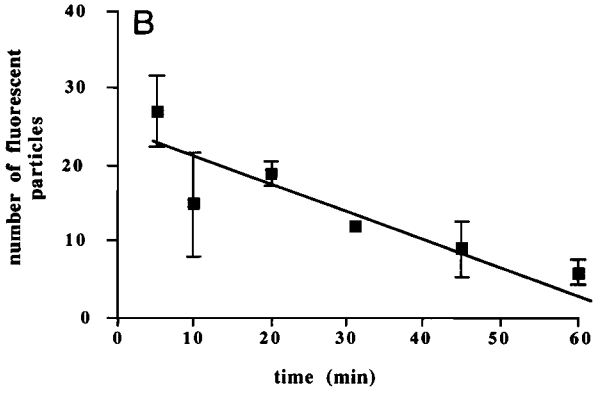

A
B
C
E
D
E'

Figure 2.

Evolution over time of the mean diameter (in μm, A), number (B) and distance to the nuclear center (in μm, C) of fluorescent endosome-like particles in fluo-NT-labeled basal forebrain neurons. Slices from the rat basal forebrain were labeled as described in Fig. 1 and scanned under the confocal microscope 5-60 min after a single 3 min pulse of 20nM fluo-NT. All measurements were made on 10μm-thick optical sections scanned at high resolution (128 scans/frame, Fig. 1c,d). Mean ± SEM of 12 slices from 3 animals. Statistical analysis was performed using a one way analysis of variance (ANOVA) followed by a regression analysis.


A
diameter of fluorescent particles (µm)
1.2
1.0
0.8
0.6
0
10
20
30
40
50
60
time (min)


B
number of fluorescent particles
40
30
20
10
0
0
10
20
30
40
50
60
time (min)

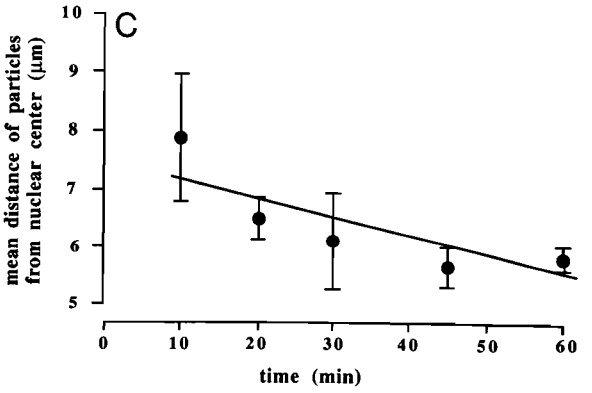

C
mean distance of particles from nuclear center (µm)
10
9
8
7
6
5
0
10
20
30
40
50
60
time (min)

Figure 3.

Confocal microscopic imaging of baculovirus-infected Sf9 cells incubated with 10nM fluo-NT for 60 min at 21°C in the absence (a) or the presence (b) of the endocytosis inhibitor, phenylarsine oxide. Single 0.42μm-thick optical sections taken at the cell's mid height and averaged over 32 scans/frame. Whereas the ligand is clearly internalized in cells labeled in the absence of phenylarsine oxide (a), it remains confined to the cell surface in cells exposed to the inhibitor. Scale bar: 10 μm.

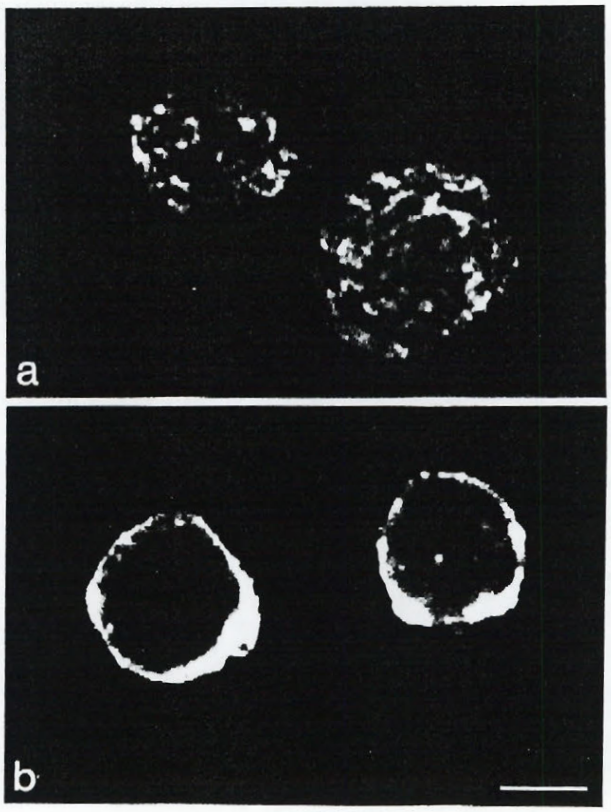

a
b

Figure 4.

Confocal microscopic imaging of the binding (a,b) and internalization (c,d) of fluo-azido-nitro NT to Sf9 cells infected with a baculovirus coding for the rat NT receptor. Images acquired as in Fig. 5. At -5°C (a,b) the label is confined to the cell surface in irradiated cells (a) and virtually undetectable in non-irradiated ones (b). At 21°C (c,d), cross-linked fluo-azido-nitro NT molecules have internalized and are detected throughout the cytoplasm in the form of intensely fluorescent particles (c). Here again, non-irradiated controls are negative (d). Scale bar: 5µm.

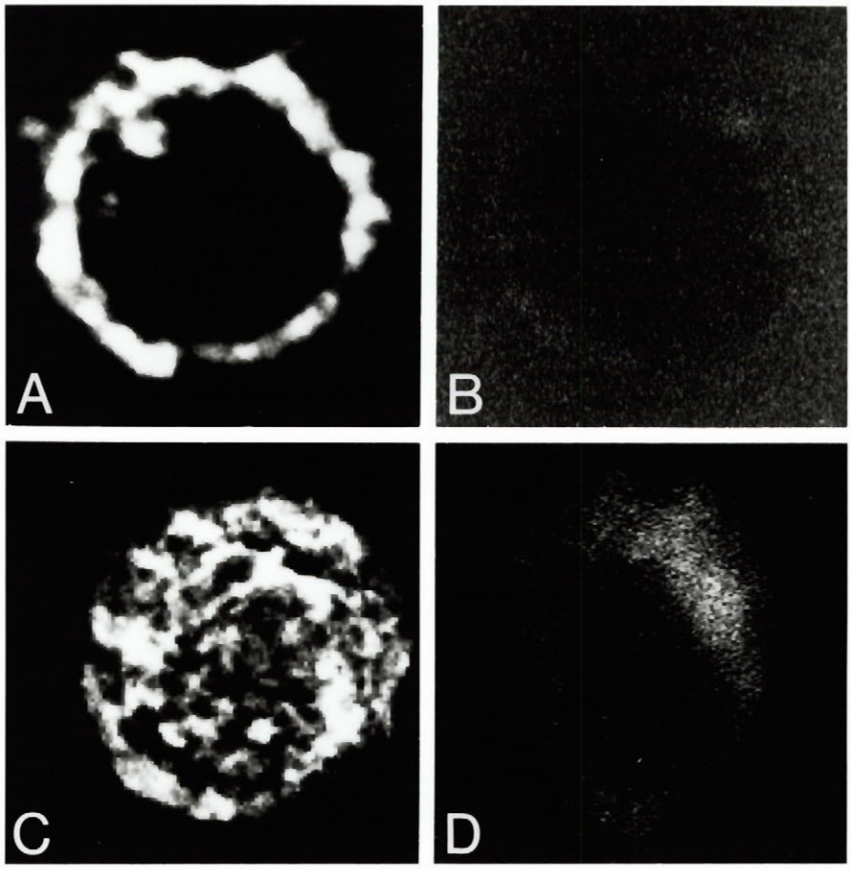

A
B
C
D

Table I: Densitometric analysis of fluorescence intensity in slices of rat basal forebrain incubated with fluo-NT.

Washout time	Fluorescence intensity*		
	perikarya	neuropil	perikarya/neuropil
5 min	85.73 ± 5.40	64.74 ± 3.79	1.52 ± 0.11
30 min	92.00 ± 7.37	55.19 ± 8.96	1.97 ± 0.23
60 min	101.94 ± 3.43	43.28 ± 5.17	2.51 ± 0.28

*Grey levels/unit area on a 0-255 scale. Values represent the mean ± SEM of 12-14 readings from at least 4 different slices. Perikaryal values inversely correlated with neuropil ones ($r=-0.99$) with $p<0.001$.

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Connecting text #2

We have demonstrated that fluo-NT is internalized in basal forebrain cholinergic neurons in *ex-vivo* brain slices, and that this internalization is receptor-mediated. Fluo-NT was also shown to be bioactive since it elicited electrophysiological effects similar to native NT.

In order to further characterize this phenomenon , the kinetics of NT internalization were studied *in vitro*.

CHAPTER 4

BINDING AND INTERNALIZATION OF NEUROTENSIN IN HYBRID CELLS DERIVED FROM SEPTAL CHOLINERGIC NEURONS

Marie-Pierre Faure[1] *, Catherine Labbé-Jullié[2], Neil Cashman[1], Patrick Kitabgi[2] and Alain Beaudet[1]

[1] Montreal Neurological Institute, 3801 University Street, Montreal, Quebec, H3A 2B4, Canada. [2] Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, UPR 0411, Université de Nice Sophia-Antipolis, 660 Route des Lucioles, Sophia-Antipolis, 06560 Valbonne, France.

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ABSTRACT

Autoradiographic studies from our laboratory have demonstrated a selective association of high affinity neurotensin (NT) binding sites with basal forebrain cholinergic neurons. In search of an *in vitro* model for further characterization of the role and regulation of these sites, we have examined the binding and internalization of ^{125}I-Tyr$_3$-NT (^{125}I-NT) and fluorescein isothiocyanate (FITC)-conjugated NT (fluo-NT) on SN17 hybrid cells, produced by fusion of embryonic murine septal cells with neuroblastoma. ^{125}I-NT binding to SN17 membrane preparations was specific and saturable. Scatchard analysis of the data was suggestive of an interaction with a single population of sites, the affinity (Kd = 1.7 nM) and pharmacological profile of which were comparable to those of neural NT receptors. No specific binding was observed on the parent neuroblastoma cell line confirming that the expression of those sites is a neuronal trait. Incubation of whole SN17 cells with ^{125}I-NT resulted in a time- and temperature-dependent internalization of the specifically bound peptide. The t½ of this internalization was estimated at 13 min, a value nearly identical to that reported for neurons in culture. Confocal microscopic analysis of the internalization process carried out using fluo-NT indicated that it was endocytic in nature in that: (1) it was entirely blocked by the endocytosis inhibitor, phenylarsine oxide and; (2) it took the form of small intracytoplasmic particles the size and maturation of which corresponded to that of endosomes. It is proposed that the expression and internalization of NT receptors by SN17 hybrid cells represent a new facet of these cells' cholinergic phenotype which makes them emenable to the study of NT interactions with cholinergic cells.

INTRODUCTION

Neurotensin (NT) is a tridecapeptide which has been documented to play a neuromodulatory role in both central and peripheral nervous systems (see Elliott and Nemeroff, 1987; Nemeroff et al., 1980, for reviews). These modulatory effects are exerted through activation of G-protein-linked high affinity receptors (Kd = 0.3-0.4 nM) (Kitabgi et al., 1977; Uhl et al., 1977; for a review, see Kitabgi et al., 1985). Different molecular forms of high affinity NT receptors, ranging between 49 and 100 kDa, have been purified from bovine, rat and mouse brain (Mazella et al., 1989; Mills et al., 1988; Miyamoto-Lee et al., 1991). It is still unclear which of these correspond to the 424 amino acid receptor proteins that were recently cloned and sequenced from rat and human brains (Tanaka et al., 1990; Vita et al., 1993). All are clearly distinct, however, from the low-affinity NT binding site (Kd = 19 nM) which has been described in rodent brain (Schotte et al., 1986). This low affinity site is characterized by its sensitivity to the antihistamine, levocabastine (Kitabgi et al., 1987; Schotte et al., 1986), and is believed to correspond to an "acceptor" rather than to a true receptor site.

In both rat and human brains, high affinity NT receptors were shown to be selectively associated with a number of chemo-specific neuronal systems. Thus, in the medial septum, horizontal and vertical limbs of the diagonal band of Broca and substantia innominata, NT receptors were found by combined receptor autoradiography and acetylcholinesterase histochemistry to be selectively associated with cholinergic neurons (Szigethy and Beaudet, 1987). In the substantia nigra and ventral tegmental area, high affinity NT binding sites were detected in selective association with dopaminergic cells (Palacios and Kuhar, 1981; Szigethy and Beaudet, 1989). In the suprachiasmatic nucleus, the bulk of NT receptors was found to overlay the perikarya of VIP-immunoreactive

neurons (Francois-Bellan et al., 1992). Finally, in a number of forebrain (Moyse et al., 1987) and brainstem (Kessler et al., 1987) nuclei, NT receptors were found to be selectively enriched in areas containing acetylcholinesterase-positive neurons. In all of these cell systems, labelled NT binding sites were heavily concentrated inside the cytoplasm of perikarya and dendrites rather than confined to the cell surface (Dana et al., 1989; Szigethy et al., 1990). This intracellular localization was in keeping with the results of histochemical, biochemical and autoradiographic studies in primary neurons in culture which had demonstrated that radiolabelled NT was internalized in a time-and temperature-dependent manner (Mazella et al., 1991; Vanisberg et al., 1991; for a review, see Beaudet et al., 1994). This internalization process has been postulated to account for desensitization and down-regulation of the receptor (Chabry et al., 1993; Donato Di Paola et al., 1993; Hermans et al., 1994; Mazella et al., 1991; Turner et al., 1990; Vanisberg et al., 1991) as well as for the degradation of bound radioligand molecules (Woulfe et al., 1992).

The aims of the present study were to establish an *in vitro* model for the visualization and kinetic analysis of NT internalization in transmitter-defined nerve cells. For this purpose, we resorted to a cell line, SN17, produced by fusion of embryonic murine septal cells with murine neuroblastoma (Hammond et al., 1986; Hammond et al., 1990). These cells had previously been studied in detail and found to express neuronal characteristics such as the formation of neurites and expression of the neurofilament proteins, but not of the glial marker, glial fibrillary acid protein (GFAP) (Hammond et al., 1986). Most importantly, these cells were shown to express the biosynthetic enzyme for acetylcholine, choline acetyltransferase (ChAT), and could therefore be expected to express other phenotypic traits of septal cholinergic cells such as high affinity NT receptors. In a first set of experiments, the binding and the internalization of NT to SN17

cells were characterized biochemically, and in a second set of experiments, the fate of the internalized ligand was monitored by confocal laser microscopy using a specific fluorescent probe.

MATERIALS AND METHODS

1-Chemicals

Unlabelled NT (1-13), NT (8-13), NT (1-8), fluorescein isothiocyanate (FITC), 1,10-phenanthroline (a protease inhibitor) and phenylarsine oxide (PheAsO: an endocytosis inhibitor) were purchased from Sigma (USA). Monoiodo-[^{125}I-Tyr$_3$]NT (^{125}I-NT) was prepared at a specific activity of 2000 Ci/mmol, as previously described (Sadoul et al., 1984). FITC-conjugated NT (fluo-NT) was synthesized as reported (Faure et al., 1994). Levocabastine (R50547) was kindly provided to us by Janssen Pharmaceutica (Beerse, Belgium) and the NT antagonist, SR 48692, by Dr. D. Guilly (Sanofi, Toulouse, France). Dubelcco's modified Eagle's medium (DMEM), fetal calf serum and penicillin-streptomycin solutions were purchased from Gibco BRL (Canada). All other reagents were of the best commercially available grade.

2-Culture conditions

Hybridoma cells, SN17, produced by the fusion of embryonic mouse septal cells with a murine neuroblastoma (Hammond et al., 1990) were generously provided by D.N. Hammond. The parent neuroblastoma N18 cells were grown in petri dishes (100 mm^2) in DMEM containing 44 mM $NaHCO_3$ and 10% fetal calf serum in the presence of 100 µU/ml penicillin and streptomycin. Cells were grown for 3 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For binding experiments, cells were plated in plastic petri dishes at a density of 0.6-1.2 x 10^6 cells per 100 mm^2. For confocal imaging of NT receptors, cells were grown as a monolayer on 18mm^2 polylysine-treated glass coverslips to 50% confluence in 35mm^2 petri dishes. At least 24 hours before all assays, cells were transferred from the above medium to DMEM containing 0.5% calf serum in order to

induce the differentiation of neuronal traits.

3-Binding of ^{125}I-NT to SN17 and N18 cell membranes

Cell membrane preparation

Cells were washed with DMEM and left undisturbed for 10 min. Culture medium was removed and cells were scraped with a rubber policeman in ice-cold 25 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM EDTA, and centrifuged at 4°C for 15 min at 30000 rpm. The cell pellet was resuspended and lysed for 30 min at 4°C in 1 ml of hypotonic buffer containing 5 mM Tris-HCL and 5 mM EDTA. The lysate was centrifuged again at 4°C for 15 min at 30000 rpm and the final pellet was resuspended in 5 mM Tris-HCl. The final homogenate was assayed for protein concentration using a Bio-Rad assay reagent, with serum albumin as a standard. The homogenate (2 mg/ml) was aliquoted and stored at -80°C until used.

Binding experiments

Homogenates of SN17 and N18 cells (100 µg of protein) were incubated in the presence of 100 pM ^{125}I-NT and increasing concentrations of unlabelled NT (0.1-100 nM), acetyl-NT (8-13), NT (1-8), levocabastine or the non-peptide NT antagonist SR 48692 in 250 µl of binding buffer (Tris-HCl 50 mM, pH 7.4, containing 0.1% bovine serum albumin, 1 nM 1,10-phenanthroline) for 20 min at 20°C in plastic tubes. Non-specific binding was determined in the presence of 1 µM of unlabelled NT. The incubation was terminated by the addition of 2 ml of ice-cold binding buffer followed by filtration under vacuum through 0.2 µm cellulose acetate membrane filters (Micro Filtration Systems, San Francisco, CA) presoaked in binding buffer for 1 h at 4°C. Tubes and filters were then washed twice with 2 ml of ice-cold binding buffer. The radioactivity retained on

the filter was measured with a gamma counter with a counting efficiency of 80%. Specific binding was obtained by subtracting non-specific from total binding. The data were expressed as the percentage of maximum specific binding observed in the absence of competitor. IC_{50} values were calculated from the competition curves thus obtained and corrected for occupancy by the labelled ligand according to the method of Cheng and Prusoff (1973) to obtain Ki values. The dissociation constant (Kd) and maximal binding capacity (Bmax) for NT were derived from Scatchard analysis of saturation experiments performed with increasing concentrations (100-500 pM) of ^{125}I-NT combined with competition experiments in which varying concentrations of unlabelled NT were added to a fixed concentration (500 pM) of ^{125}I-NT. All binding data were analyzed on a microcomputer using ABDA/LIGAND programs.

4-Binding of ^{125}I-NT to intact and N18 cells

Time course experiments

For this set of experiments, the culture medium was removed, the cells were washed twice with fresh culture medium devoid of fetal calf serum and once with Earle's buffer made of 50 mM Hepes-Tris buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.8 M $CaCl_2$, 3.6 mM $MgCl_2$, 0.2% bovine serum albumin, 6 g/l glucose and 1 mM of the peptidase inhibitor, 1,10-phenanthroline. The cells were harvested by agitation, collected by centrifugation at 1500 g for 5 min at 22°C and allowed to rest for 10 min before initiating the binding. Binding experiments were performed at either 4°C or 37°C, at equilibrium. SN17 or N18 cells (500 000) were incubated with 0.2 nM ^{125}I-NT in 1 ml of binding buffer, in the presence or absence of 1 µM NT to determine non specific binding. Additional experiments were carried out in the presence of 1 µM levocabastine to block low affinity NT binding sites. At various time points (10 min to 60 min) after incubation,

the cells were centrifuged for 5 min at 1500 g, and the binding medium was removed. Cells were rapidly filtered on 0.2 μm presoaked filter and washed three times with 2.5 ml of ice-cold washing buffer. The radioactivity bound to the cells was measured with a gamma counter.

Differentiation of internal versus cell surface binding sites

To differentiate internalized from surface-bound ^{125}I-NT, cells were incubated as above with 0.2 nM ^{125}I-NT for 60 min at 4°C. They were then rapidly washed, resuspended in binding buffer and warmed up to 37°C. The incubation was stopped 5-60 min later by two sequential ice-cold buffer washes. Surface-bound radioactivity was then released by the addition of 1.5 ml of ice cold 0.1 M acetic acid in 0.5 M NaCl, pH 2.5 (Haigler et al., 1980) for 4 min at 4°C. The acid wash was then collected and the radioactivity measured with a gamma counter. The radioactivity present in the remaining pellet was also measured as an index of intracellular ^{125}I-NT. The rate constant, Ke, for ^{125}I-NT internalization was calculated as described by Wiley and Cunningham (1982). Non-specific binding was measured by incubating additional samples in the presence of a 100-fold excess of unlabelled NT.

5-Confocal microscopic studies

SN17 and N18 cells were rinsed twice with TBS to remove any trace of medium and equilibrated with Earle's buffer and 1 mM of the peptidase inhibitor, 1,10-phenanthroline, for 10 min at 4°C, in the presence or absence of 10 μM of PheAsO (Chabry et al., 1993). Pre-incubation buffers were removed and the cells incubated for 60 min at 4°C in Earle's buffer containing 20 nM of the fluorescent NT agonist fluo-NT (Faure et al., 1994). The cells were then rapidly washed, and either rinsed in cold

phosphate-buffer-saline (4 x 60 sec) or transferred to 37°C for 5, 15, 30 or 60 additional min. Some of the cells were incubated according to the same protocol, but in the presence of a 1000 fold excess of non-fluorescent NT for determination of non-specific binding. All cell preparations were then air dried under a cool stream of air in the dark and examined under a Leica confocal laser scanning microscope (CLSM). The instrument consisted of a Leica Diaplan inverted microscope equipped with an argon ion laser (488 nm for FITC excitation) with an output power of 2-50 mV and linked to a VME bus MC 68020/68881 computer coupled to an optical disc for image storage (Leica, St. Laurent, Canada). The emission filter was a highpass from 550 nm. The pictures were taken from the image monitor using a Focus Image Corder (Focus, Los Angeles, CA).

Twenty five serial 0.12 μm-thick optical sections (512x512 pixels) were collected per scanning sequence. Images were acquired at a scan rate of 4 sec/scan and averaged over 32 scans/frame for optimal sensitivity. The gain and black levels were set manually to optimize the dynamic range of the image while ensuring that no region was completely suppressed (intensity = 0) or completely saturated (intensity = 255). The profile of fluorescence intensity was measured along a single line passing through the cell and the results expressed as grey level units on a 0-255 grey scale.

RESULTS

Characterization of ^{125}I-NT binding to SN17 and N18 membrane preparations

It was observed in preliminary experiments that the specific binding of 0.1 pM ^{125}I-NT to SN17 cell homogenates increased with time and reached a plateau by 10-15 min whereas non-specific binding remained constant over time and never exceeded 5% of total binding (not shown). In subsequent experiments, ^{125}I-NT binding was measured after a 20 min incubation. There was no detectable specific ^{125}I-NT binding on homogenates from N18 cells, indicating that the parent neuroblastoma cell line was devoid of high affinity NT binding sites.

As illustrated in Fig. 1, unlabelled NT inhibited specific ^{125}I-NT binding to SN17 cell homogenates in a concentration-dependent manner with a Ki value of 2.3 ± 0.32 nM (mean ± SEM from three experiments). When NT binding data from combined saturation and competition experiments were subjected to Scatchard analysis, a linear best fit was obtained (Fig. 2) indicating that NT bound to a single population of sites with Kd and Bmax values of 1.7 ± 0.48 nM and 226.7 ± 18.7 fmol/mg protein, respectively. The fact that the Kd and Ki values were in good agreement indicated that labelled and unlabelled NT had bound with equal affinity to SN17 homogenates.

The C-terminal NT fragment acetyl-NT (8-13) and the non-peptide NT antagonist SR 48692 inhibited ^{125}I-NT binding to SN17 cell membrane homogenates in a concentration dependent fashion with respective Ki values of 2.5 ± 0.43 nM and 25.2 ± 2.82 nM (mean ±SEM from three experiments) (Fig. 1). By contrast, the N-terminal NT fragment (1-8) and the antihistamine levocabastine were devoid of effect on ^{125}I-NT binding at concentrations of up to 30 μM (Fig. 1).

Binding and internalization of ^{125}I-NT in whole SN17 cells

The effect of temperature on the association of ^{125}I-NT to whole SN17 cells is illustrated in Fig. 3. At 4°C, specific ^{125}I-NT binding increased slowly with time and reached a plateau between 40 and 60 min. At 37°C, the association of ^{125}I-NT to SN17 cells was more rapid and reached a higher plateau value than at 4°C.

The evolution of acid-resistant (i.e., intra-cellular) versus acid-washable (i.e., surface-bound) radioactivity in cells warmed to 37°C after labelling of their cell surface receptors at 4°C is illustrated in Fig. 4. The proportion of ^{125}I-NT recovered in the acid-resistant fraction increased markedly over the first 15 min and more progressively thereafter to reach approximately 80% of specifically bound radioactivity (Fig. 4). Conversely, the proportion of ^{125}I-NT recovered in the acid-washable fraction decreased progressively with time to approximately 20% of specifically bound ^{125}I-NT (Fig. 4). Over the 60 min duration of the experiment, the total amount of bound ^{125}I-NT remained virtually constant, suggesting that ligand degradation had been negligible during this period (Fig. 4). The integrity of the cells was not affected by the rapid low-pH treatment, as judged by their ability to exclude Trypan Blue (data not shown). As can be seen in Fig. 5, the ratio of internalized to surface bound ligand increased as a function of time. The rate constant for internalization, Ke, as derived from the slope of this curve was of 0.94 ± 0.10 min^{-1} (SD, n = 7) and the t½ of 12.69 min.

Confocal imaging of NT internalization

The labeling pattern of SN17 hybrid cells with the fluorescent NT derivative (agonist), fluo-NT varied markedly with the temperature of incubation. After incubation at 4°C, virtually all SN17 cells exhibited numerous intensely fluorescent hot spots, 0.6-0.9 µm in diameter, over their perikarya and processes (Fig. 6a, a'). Serial optical sectioning

of labelled cells demonstrated that these hot spots were mainly confined to the cell surface (Fig. 6b, b'). Quantification of the fluorescent signal yielded a mean fluorescence intensity of 195 ± 22 per hot spot (on a 0-255 grey scale; Fig. 6a'). It also showed that while the labelling intensity was homogeneous over small hot spots, it was heterogenous over large ones, suggesting that the latter corresponded to clusters of hot spots rather than to independent entities (Fig. 6a').

Cells transferred at 37°C after saturation of cell surface receptors also exhibited intense, heterogenous fluorescent labelling. However, in contrast to the pattern observed at 4°C, the labelling at 37°C was found by serial optical sectioning to be inside the cytoplasm of the cells (Fig. 7). After 5 min of incubation, the labelling took the form of numerous small fluorescent granules, 0.2-0.4 μm in diameter, prominent against a relatively high diffuse fluorescence in background (Fig. 7a). The mean intra-particle fluorescence intensity was 101 ± 8 against a background fluorescence of 86 ± 5. At 15 min, the labelling appeared more distinctly punctuate because of the concomitant increase in intragranular fluorescence intensity (110 ± 9) and the decrease in background signal (64 ± 9) (Fig. 7b). At 30 min, the fluorescence intensity within intracytoplasmic granules was up to 132 ± 12 and the background intensity was down to 55 ± 3 (Fig. 7c). The diameter of the fluorescent granules was also larger than at shorter time intervals (0.5-1.5 μm in diameter; Fig. 7d). After 60 min of incubation, the intracellular labelling was still granular in appearance but the fluorescent particles were both larger (1.5-2 μm) and more intensely labelled (202 ± 18) than at 30 min (Fig. 7d). No clearcut nuclear labeling was evident at any of the times examined.

SN17 hybrid cells treated with PheAsO prior to incubation with fluo-NT showed no internalization of the label, even after 60 min of exposure at 37°C. At that time, the bound fluorescent ligand formed sparse large hot spots (2.5-3 μm^2) over the cell surface

(Fig. 8).

SN17 cells incubated in the presence of an excess of NT (1-13) at either 4°C or 37°C exhibited no fluorescent labelling.

DISCUSSION

The present study demonstrates that SN17 hybrid cells express specific high affinity NT binding sites and that these sites provide for a rapid internalization of the peptide as they do in basal forebrain cholinergic neurons (Alonso et al., 1994).

^{125}I-NT binding to SN17 membrane preparations was specific and saturable. Scatchard analysis of the data was suggestive of an interaction with a single population of sites with a Kd of 1.7 nM. This value is comparable to that previously obtained in other murine neuronal cell lines including differentiated neuroblastoma cells N1E-115 (Amar et al., 1987; Bozou et al., 1986; Gilbert et al., 1986; Poustis et al., 1984), neuroblastoma x glioma cells NG 108-15 (Nakagawa et al., 1984) and rat embryonic neurons (Brouard et al., 1992; Checler et al., 1986; Mazella et al., 1993; Vanisberg et al., 1991).

NT binding sites associated with SN17 cells were levocabastine insensitive and thus clearly distinct from the so-called low affinity acceptor site previously described in murine brain (Schotte et al., 1986). No specific binding was observed on membranes from the parent neuroblastoma cell line N18, suggesting that the expression of the high affinity NT receptor by SN17 cells is a property derived from septal neurons.

NT and acetyl-NT (8-13) inhibited ^{125}I-NT binding with potencies comparable to those reported by others in primary neuronal cultures (Checler et al., 1986). As previously recognized (Kitabgi et al., 1980), the C-terminal fragment of NT, acetyl-NT (8-13), was equipotent with NT in competiting for ^{125}I-NT binding, whereas the N-terminal portion of NT, NT (1-8), was inactive. The NT antagonist, SR 48692, also competed with ^{125}I-NT binding with a Ki value similar to that observed in mouse brain homogenates (Gully et al., 1993).

In sum, the present data demonstrate that SN17 hybrid cells possess a single

population of high affinity NT binding sites, the pharmacological characteristics of which are comparable to those of central NT receptors (Kitabgi et al., 1985). They also suggest that the expression of these sites is a neuronal trait, which conforms to the reported association of high affinity NT receptors with septal neurons (Moyse et al., 1987). Given that the vast majority of septal neurons endowed with high affinity NT binding sites were shown to be cholinergic (Szigethy and Beaudet, 1987), the expression of these sites by SN17 cells may be viewed as another facet of their cholinergic phenotype (Hammond et al., 1986).

As previously observed in primary cultures of neurons (Mazella et al., 1991; Mazella et al., 1993), the maximal number of sites labelled with ^{125}I-NT on whole SN17 cells was higher at 37°C that at 4°C. This temperature-dependent increase in specific ^{125}I-NT binding was previously shown to be due to concomitant internalization of bound ligand molecules (Mazella et al., 1991; Vanisberg et al., 1991) and surface translocation of intracellular spare receptors (Chabry et al., 1993). Two distinct lines of evidence, biochemical and morphological, indicate that similar mechanisms are operational on SN17 cells.

First, the results of our acid wash experiments indicated that the acid-washable fraction of specifically bound ^{125}I-NT decreased with time proportionally to the increase in intracytoplasmic radioactivity, indicating that the surface-bound radioactivity had internalized within the cells. This internalization process was temperature-dependent since the radioactivity detected at 4°C remained membrane-bound at all times. The t½ of internalization, as derived from the ratio of surface vs internal radioactivity, was estimated at 13 min, a value similar to that previously observed for ^{125}I-NT in neurons in culture (Mazella et al., 1993).

That bound NT molecules were indeed internalized in SN17 cells was further

confirmed here by confocal laser microscopy using fluo-NT, a fluoresceinylated NT conjugate, as NT receptor marker. This compound was previously shown by us to display both the pharmacological and the electrophysiological properties of native NT and to provide for selective *in vitro* labelling of high affinity NT receptors (Alonso et al., 1994; Faure et al., 1994). Following incubation of SN17 cells at 4°C, specifically bound fluo-NT was confined to the cell surface, in agreement with our biochemical observations. By contrast, as soon as fluo-NT-labelled cells were warmed to 37°C, the label became apparent throughout the cytoplasm of the cells.

In cells labelled at 4°C, bound fluo-NT molecules were not homogeneously distributed throughout the cell surface, but rather formed small, irregular hot spots. Both ligand-gated channels (Craig et al., 1993; Nicola et al., 1992; Velazquez et al., 1989) and G-protein-coupled neurotransmitter receptors (Hazum et al., 1980a; Hazum et al., 1980b; Lutz et al., 1990; Naor et al., 1981; Raposo et al., 1987) have previously been reported to form patches or hot spots on the surface of neuronal and/or epithelial cells in culture. However, in the case of ligand-gated channels, such as glutamate (Craig et al., 1993), GABA A (Velazquez et al., 1989) and glycine (Nicola et al., 1992) receptors, these hot spots represent pre-formed clusters of receptors that are anchored to the cytoskeleton, whereas in the case of G-protein-linked receptors, such as muscarinic (Raposo et al., 1987), ß-adrenergic (Raposo et al., 1989) or peptide (Hazum et al., 1980a,b; Lutz et al., 1990; Naor et al., 1981) receptors, these hot spots are believed to be the result of a ligand-induced clustering of receptors that were initially free to diffuse laterally in the plane of the membrane. The clusters of NT receptors observed here on SN17 cells are likely to be ligand-induced rather than pre-formed given that their size increased and their number decreased when internalization was prevented to occur by pretreating the cells with PheAsO. The fact that this receptor clustering could be observed even at 4°C is probably

mainly due to technical factors, namely to the difficulty of maintaining the cells at such low temperature until confocal microscopic viewing. Furthermore, a degree of plasma membrane fluidity has been shown to remain at 4°C (Frye and Edidin, 1970; March and Helenius, 1980).

In all systems studied thus far, cell surface aggregation of receptors was found to ultimately result in internalization of receptor-ligand complexes by endocytosis. That this is also the case for NT receptors on SN17 cells was demonstrated here by the fact that fluo-NT internalization could be entirely blocked by the endocytosis inhibitor, PheAsO. Receptor-mediated endocytosis is usually carried out through clathrin-coated pits. However, both muscarinsic (Raposo et al., 1987) and ß-adrenergic (Raposo et al., 1989) receptors have been reported to internalize via small vesicles (50-70 nm) lacking a clathrin coat. Although further studies are clearly needed to identify the exact nature of NT receptor endocytic compartments in SN17 cells, it is interesting to note that endocytic vesicles the size of the uncoated vesicles referred to above would fall below the resolution of the confocal microscope and could therefore account for the intracellular diffuse "background" fluorescence observed at short time intervals after transfering fluo-NT-labelled cells at 37°C. Furthermore, this "background" fluorescence was found to decrease with time in parallel with an increase in particulate fluorescence, as would be expected if these small vesicles were to fuse and give rise to endosome-like structures, as demonstrated in the case of muscarinic and ß-adrenergic receptors (Raposo, 1987; 1989). In fact, the diameter of intracytoplasmic fluorescent granules (0.2-0.4 μm) observed here at short time intervals (5-15 min) closely approximates that reported in other cell types for early endosomes (Helenius et al., 1983; March et al., 1986). At later time points, these fluorescent granules were found to increase in size, here again fulfilling the predicted

model of receptor endocytosis according to which early endosomes merge to form, by a remodelling process, late endosomes, multivesicular bodies and, ultimately, lysosomes (Stoorvogel et al., 1991; Dunn and Maxfield, 1992). At none of the time points examined did the internalization compartment assume a tubular appearance of the type described in epithelial mammalian cells by Hopkins et al. (1990).

The functional significance of the internalization of NT demonstrated here within SN17 cells remains a matter of speculation. The relatively high rate at which this process was found to occur suggests that one of these functions might be the removal/inactivation of bound NT. Another likely function, assuming that, as demonstrated in neurons (Chabry et al., 1993), the internalization process involves receptor-ligand complexes, would be the down regulation, degradation and/or recycling of the receptors. Finally, the internalization process might trigger in itself intracellular effects. Indeed, internalization of iodinated NT within the rat nigrostriatal neurons has been purported to account for the increase of tyrosine hydroxylase expression observed in the substantia nigra following injection of the iodinated peptide into the neostriatum (Burgevin et al., 1992).

In conclusion, the present results suggest that SN17 hybrid cells express and internalize high affinity NT receptors in a manner akin to basal forebrain cholinergic neurons and that they may therefore constitute a valid model for the study of the cellular interactions of NT with these cells

FIGURE LEGENDS

Figure 1

Inhibition of specific ^{125}I-NT binding to SN17 membrane preparations by increasing concentrations of unlabelled NT (1-13) (▲), acetyl-NT (8-13) (●), NT (1-8) (■), levocabastine (↓) and SR 48962 (♦). IC_{50}'s: NT (1-13): 5.1 ± 0.64 nM; acetyl-NT (8-13): 4.8 ± 0.57 nM. Data represent the mean of three separate experiments.

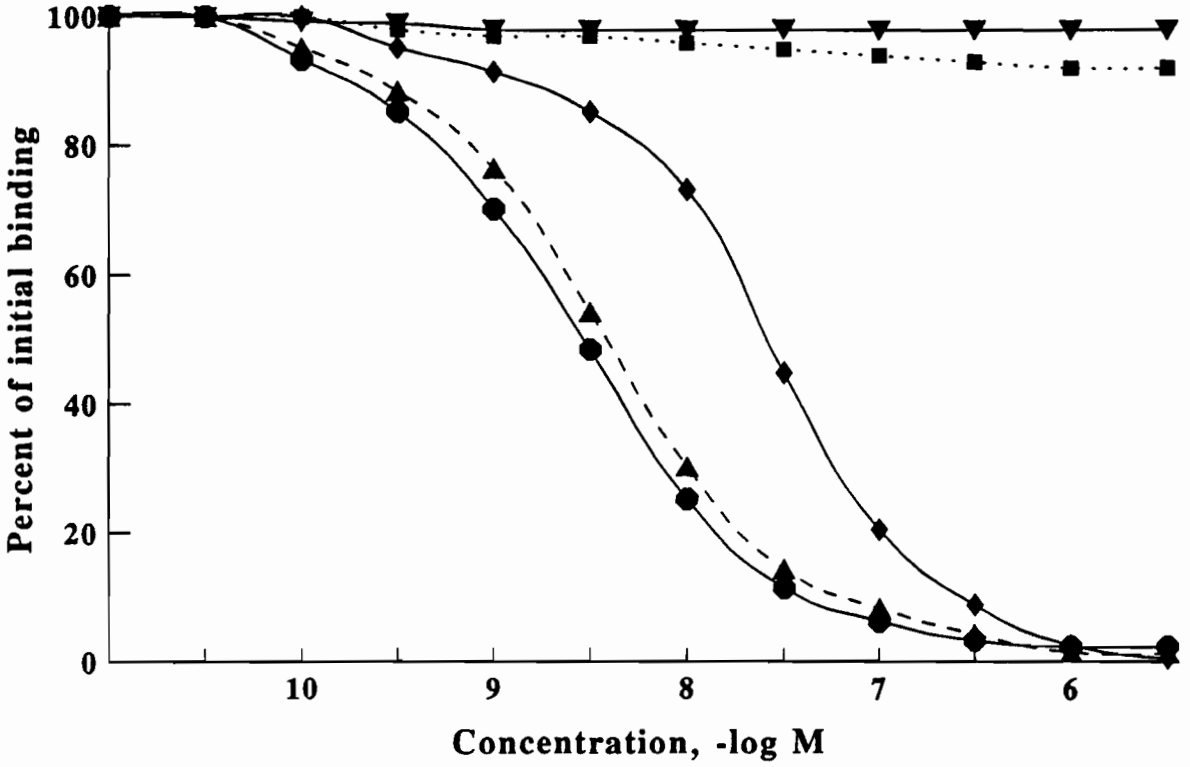

Percent of initial binding
100
80
60
40
20
0
10
9
8
7
6
Concentration, -log M

Figure 2

Scatchard analysis of ^{125}I-NT binding to SN17 membrane preparations. Data derived from combined saturation and competition experiments. B and F correspond to bound and free concentrations of ^{125}I-NT, respectively. Data are from a typical experiment with triplicate determinations that varied by less than 5%.

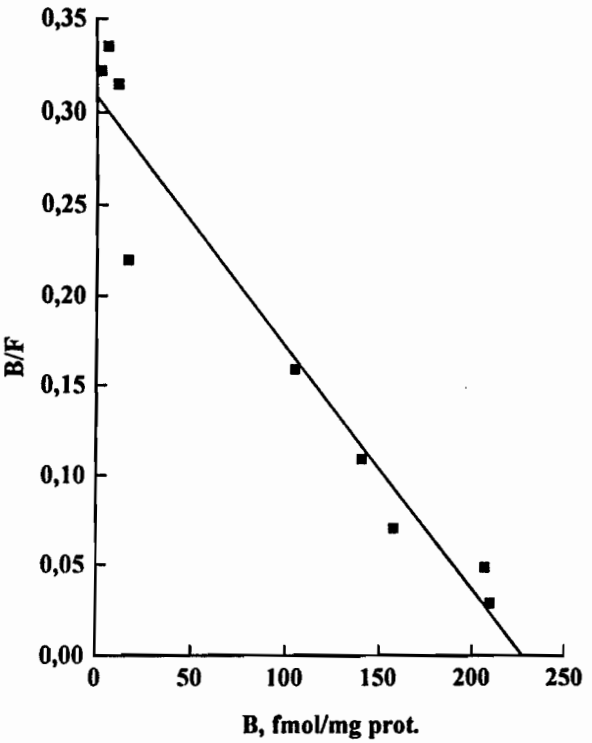

0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
B/F
0
50
100
150
200
250
B, fmol/mg prot.

Figure 3

Association of ^{125}I-NT (2 nM) to whole SN17 cells at 4°C (♦) and 37°C (•) as a function of time. Specific binding defined as the difference between binding in the absence and the presence of 1 μM NT. Each point is the mean of four determinations in two different experiments. Data expressed as cpm/assay. SEM < 5%.

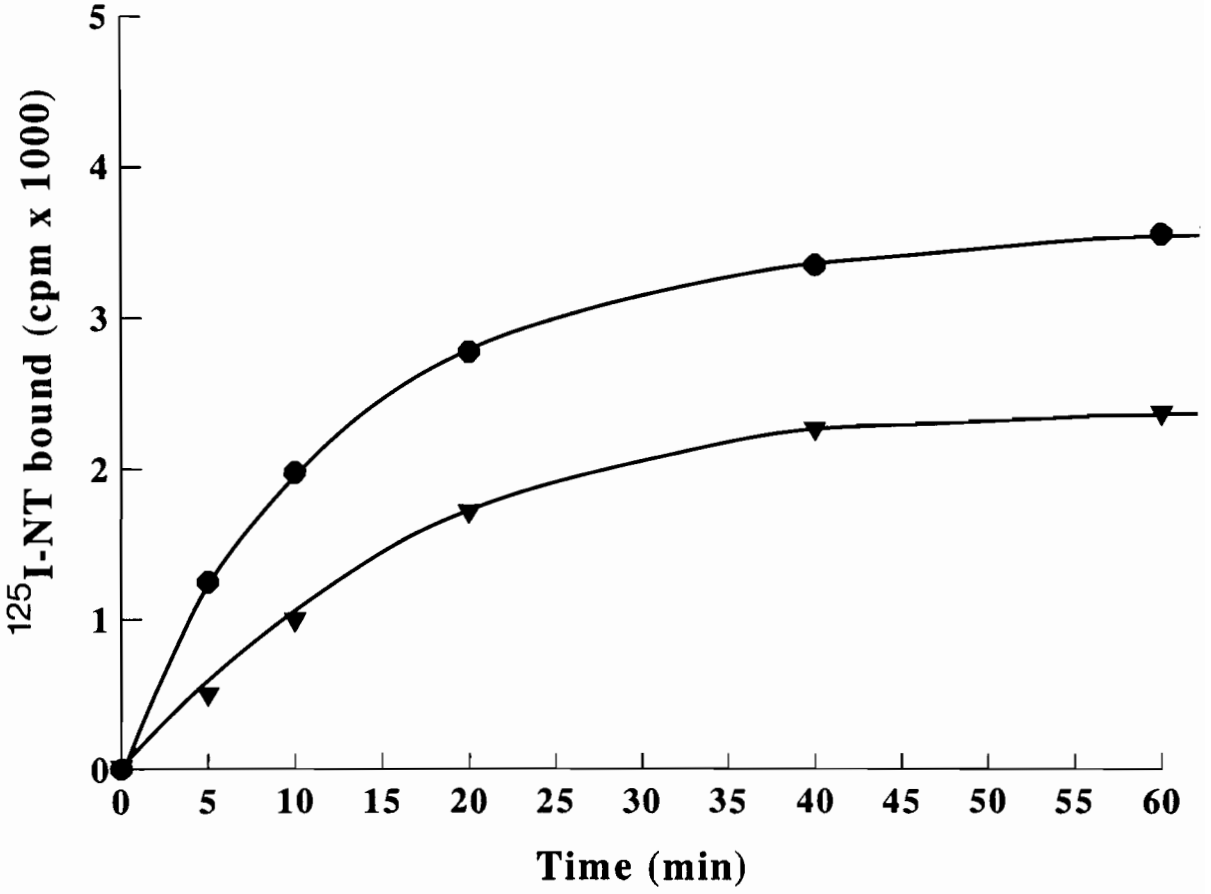

^{125}I-NT bound (cpm x 1000)
0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)

Figure 4

Kinetics of ^{125}I-NT internalization into whole SN17 cells. Cells were pre-incubated for 60 min at 4°C in the presence of 0.4 nM ^{125}I-NT, washed and warmed up to 37°C for an additional 5-60 min. The reaction was stopped by two sequential acid washes. At each time point, the acid-washable fraction of bound ^{125}I-NT (▪ ; taken to represent surface-bound radioactivity) is compared to the acid-resistant one (♦ ; corresponding to internalized ligand). Total specific ^{125}I-NT binding (•) corresponds to the sum of the two fractions. Data are the mean ± SEM of one experiment performed in triplicate.

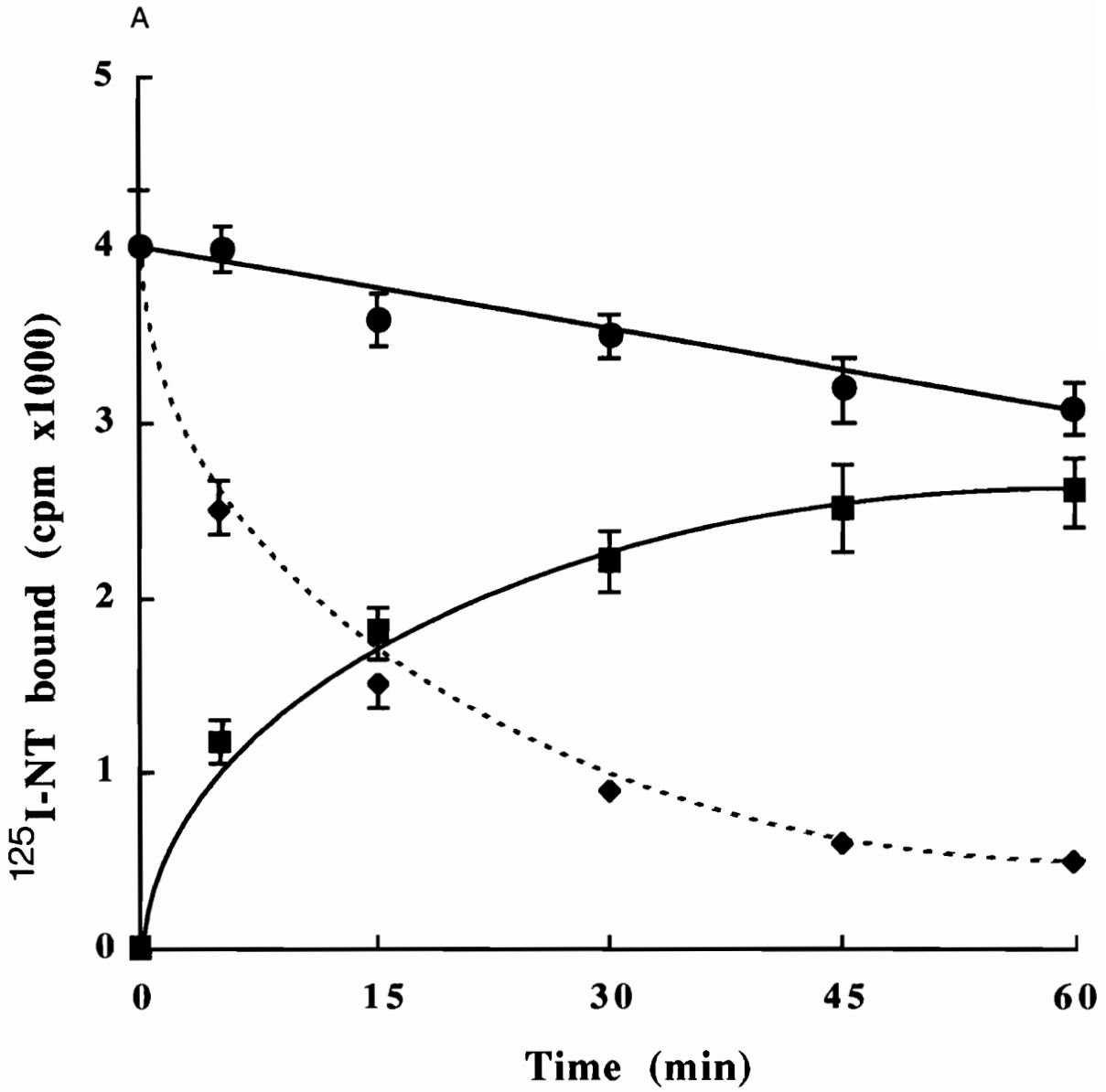

A
5
4
3
2
1
0
^{125}I-NT bound (cpm x1000)
0
15
30
45
60
Time (min)

Figure 5

Ratio of acid-resistant over acid-washable fractions plotted against time. The slope of the curve yields a rate of internalization (Ke) of 12.69 min^{-1}, with a correlation coefficient of 0.96. The data correspond to the mean of a typical experiment performed in triplicate. SEM <5%.

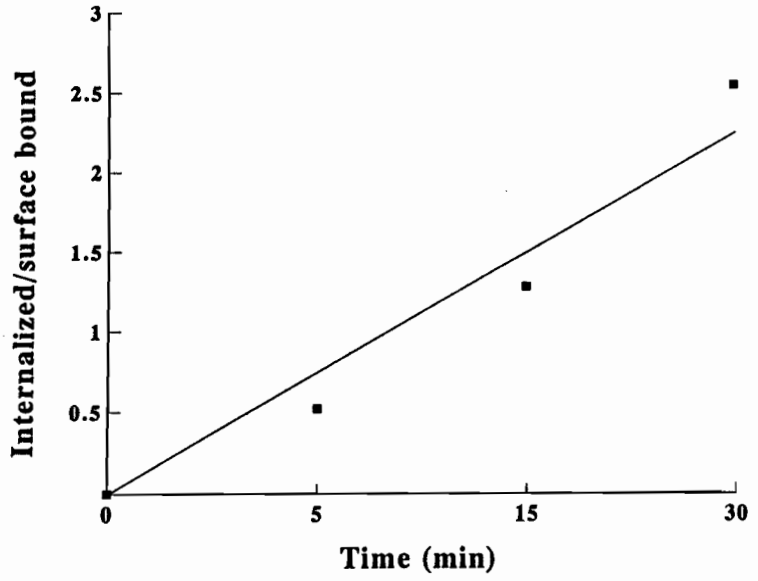

Internalized/surface bound
3
2.5
2
1.5
1
0.5
0
5
15
30
Time (min)

Figure 6

Confocal optical imaging of SN17 hybrid cells incubated for 60 min at 4°C with 20 nM fluo-NT.

a, a': Look-through projection of 25 optical sections (0.12 µm-thick) showing the distribution of the label over the cell surface. Note the presence of hot spots both over the cell body (arrows) and along a slender process outlined by arrowheads (broken arrow). In a', the same cell is overlaid with the fluorescence intensity profile (upper trace) measured along a line (lower trace) passing through the three "hot spots" identified by arrows in a.

b, b': Optical section passing through the nucleus (N) of another fluo-NT-labelled cell. b: Single optical section (0.12 µm-thick; section no. 3 out of 25). "Hot spots" of fluo-NT labelling (arrows) are apparent along the plasma membrane. b': Look-through projection of 12 optical sections (0.12 µm-thick) from the same cell. The same three hot spots as in b (arrows) are recognized in 3-dimensional rendering. Bars: 10 µm.

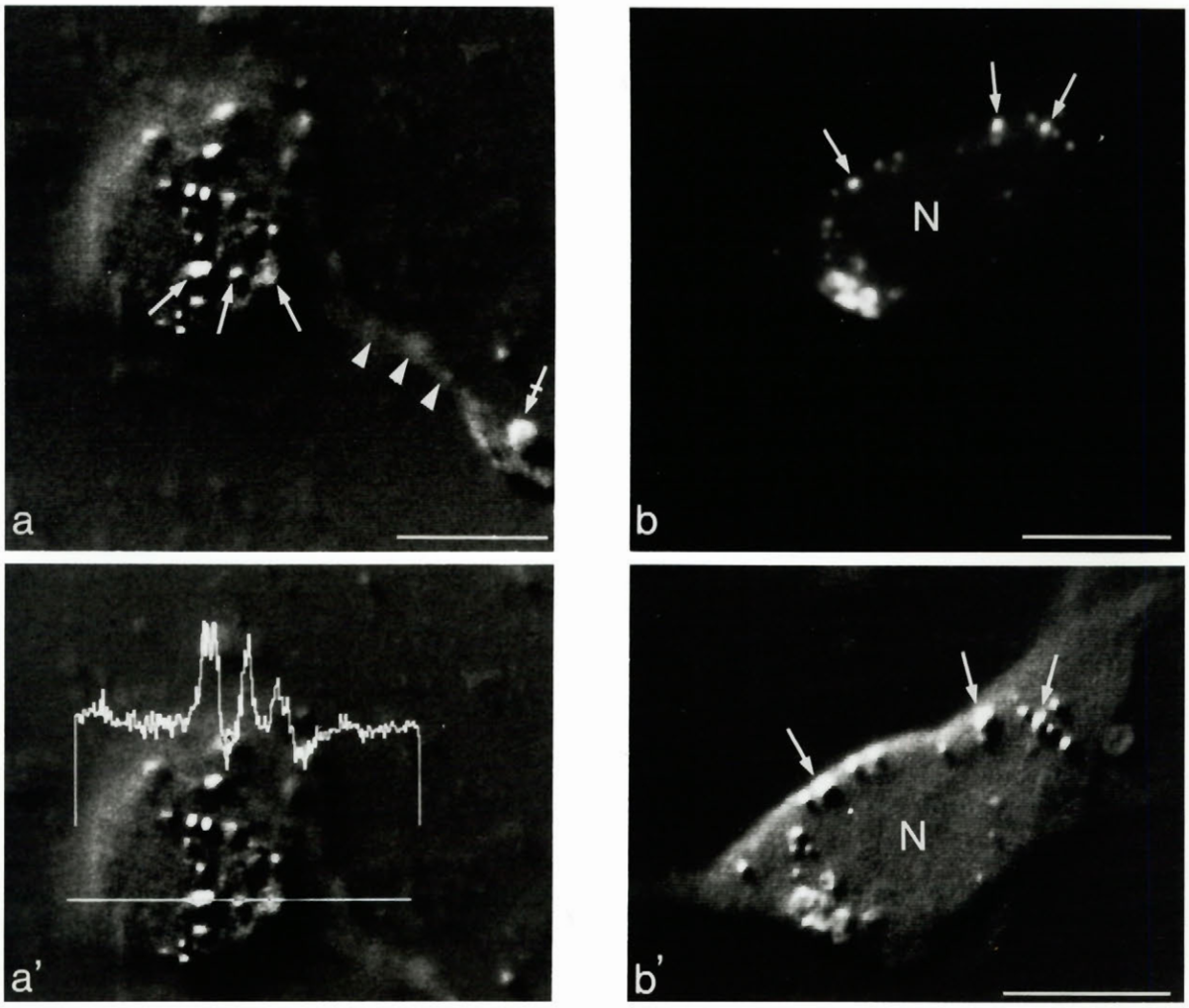

a
b
N
a'
b'
N

Figure 7

Confocal microscopic distribution of internalized fluorescence in SN17 cells incubated with fluo-NT for 1 h at 4°C and warmed up to 37°C for 5 (a), 15 (b), 30 (c) and 60 min (d). Confocal fluorescence images of 0.12 µm-thick optical sections passing through the center of the cells. At 5 and 15 min, the label is diffusely distributed throughout the cytoplasm of perikarya and processes (arrowheads), both within and outside small spherical granules (a,b). At longer time intervals, the labelling becomes progressively more granular and more intense in the perikaryon as well as in the processes (c,d). Note that the nuclear labelling is virtually exclusively confined to cytoplasmic invaginations. Bars: 10 µm.

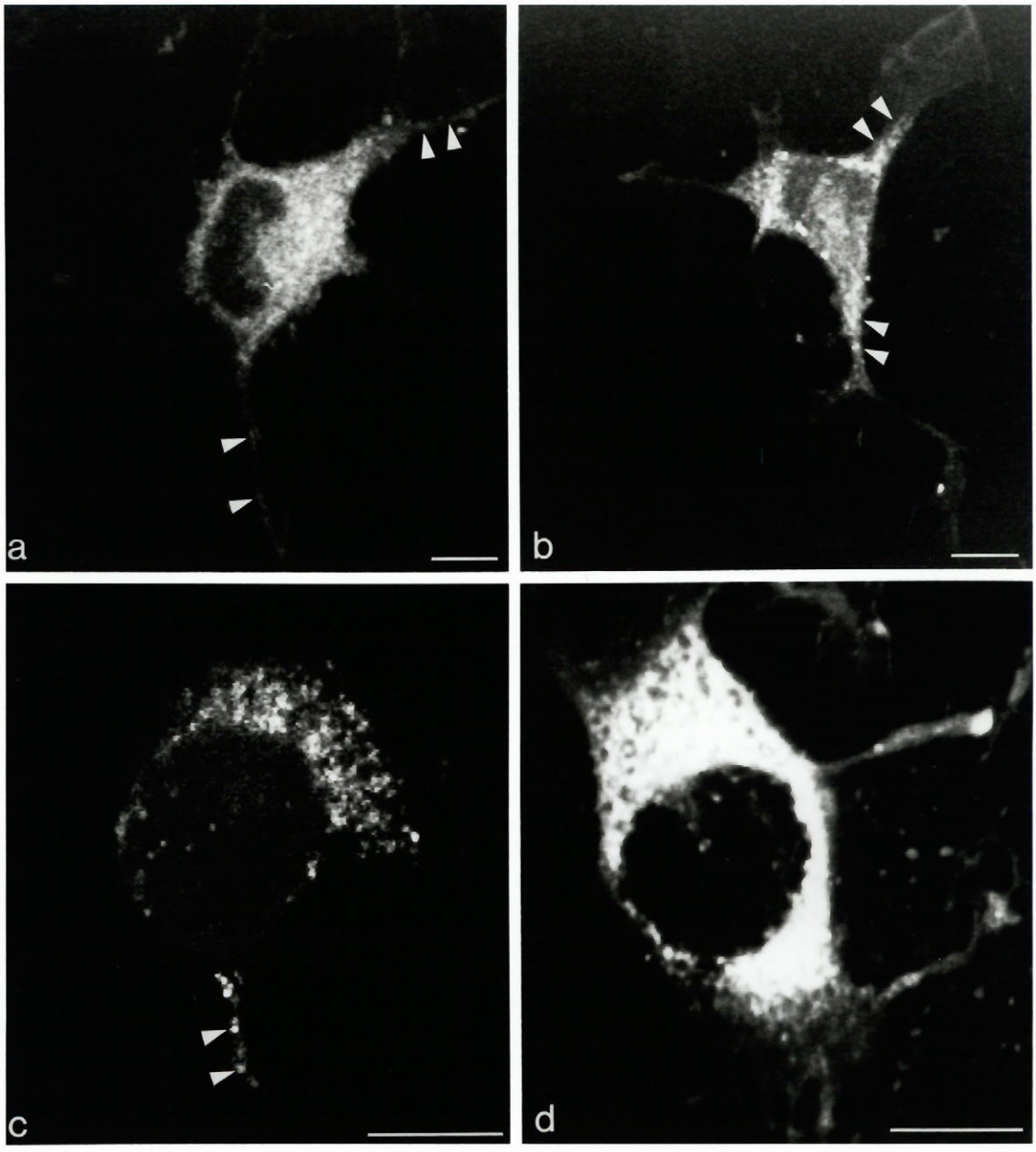
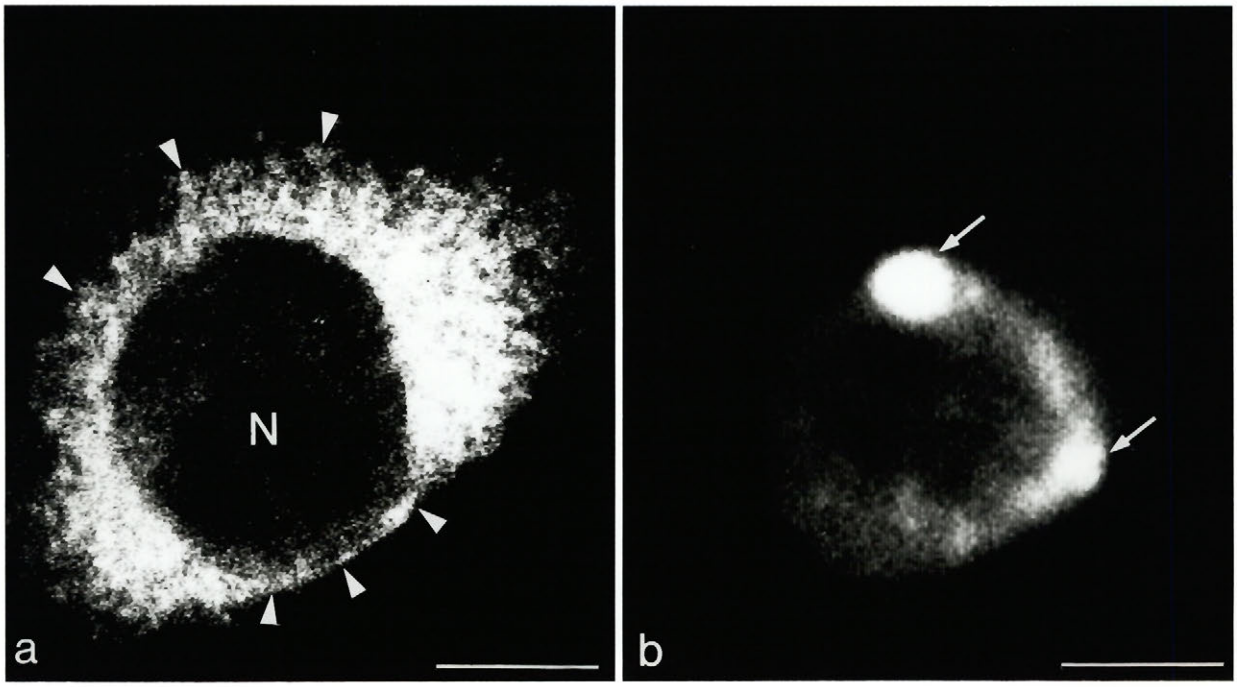

a
b
c
d

Figure 8

Effect of the endocytosis inhibitor phenylarsine oxide (PheAsO) on the internalization of fluo-NT in SN17 cells. Confocal fluorescence images collected as in Fig. 7. Cells were preincubated without (a) or with (b) PheAsO for 10 min prior to fluo-NT labelling. The labelling conditions were as in Fig. 7d. In cells pretreated with PheAsO, the fluorescent label formed large clusters on the cell surface (b; arrows), whereas in the absence of PheAsO, the labelling pervaded the cell body and was no longer apparent on the cell surface (outlined by arrowheads). Bars: 10 μm.


N
a
b

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Connecting text #3

We have shown that NT is internalized in cholinergic neurons in a time- and temperature- dependent fashion, from the somatodendritic membrane.

Since it is well known that NT exerts both short and long term effects on dopaminergic neurons and that these neurons are endowed with NT-R, it was therefore important to ascertain whether a similar phenomenon occurred in dopaminergic neurons.

CHAPTER 5

AXONAL AND DENDRITIC TRANSPORT OF INTERNALIZED NEUROTENSIN IN RAT NIGROSTRIATAL DOPAMINERGIC NEURONS

Marie-Pierre Faure, Dominique Nouel and Alain Beaudet

Montreal Neurological Institute, McGill University,
3801 University Street, Montreal, Quebec, H3A 2B4, Canada

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ABSTRACT

Previous studies have demonstrated that neurotensin is internalized and retrogradely transported in neurons of the substantia nigra following its intracerebral injection in the neostriatum. The aim of the present study was to compare the intracellular distribution of retrogradely transported material with that observed following internalization of the peptide at the somatodendritic level and to confirm that the internalization was confined to dopamine neurons. To document somatodendritic internalization, slices (350 μm) from the rat ventral midbrain were incubated *in vitro* with 20 nM fluo-NT, a fluorescent derivative of neurotensin, and immunostained 5-60 min later for tyrosine hydroxylase. To document retrograde transport, rats were injected with the same compound into the neostriatum and the brains processed for tyrosine hydroxylase immunohistochemistry 4.5 and 8 hours later. Confocal laser microscopic examination of superfused slices revealed that fluo-NT was internalized at the level of the perikarya and processes of neurons in the substantia nigra, ventral tegmental area and interfascicular nucleus. At short time intervals, the label was detected in the form of small, intensely fluorescent particles distributed throughout the cytoplasm of the cells. At longer time intervals, these fluorescent particles were larger, less numerous and confined to the perikarya where they eventually clustered against the nucleus. Following intrastriatal injection of fluo-NT, retrogradely labeled cells were apparent throughout the substantia nigra, pars compacta, as well as in the lateral part of the ventral tegmental area. Here again, the label took the form of small fluorescent particles, comparable in size, shape and distribution to those detected following superfusion of midbrain slices. In both labeling conditions, fluo-NT was strictly confined to tyrosine hydroxylase-immunoreactive cells. These results indicate that neurotensin is internalized throughout the terminal and dendritic

arborizations of mesostriatal dopamine cells and that the internalized peptide is transported centripetally from both locations to the soma of the cells. The clustering of fluorescent particles in the perinuclear region of the cells further suggests that the internalized process may play a role in long term trans-cellular signalling.

INTRODUCTION

Specific high-affinity binding sites for the tridecapeptide, neurotensin (NT), have been identified throughout the mammalian brain where they have been shown to display a selective association with certain neuronal populations. In the midbrain of the rat, NT binding sites have been localized by autoradiography to dopaminergic (DA) neurons in the substantia nigra, pars compacta (SNC) and ventral tegmental area (VTA)[34,42]. More recently, *in situ* hybridization studies have demonstrated that SNC and VTA neurons express the mRNA encoding the NT receptor, supporting the view that the high affinity binding sites vizualized by autoradiography correspond to a functional receptor[12,30,39]. Electron microscopic studies revealed that NT receptors were more or less uniformly distributed along the somatodendritic plasma membrane of midbrain DA neurons[8] and cytotoxic lesion studies indicated that they were also present on the axon terminals of these cells in the neostriatum and the nucleus accumbens[21,38]. Accordingly, application of NT into the ventral midbrain was shown to increase the electrical activity of DA neurons[37,41] and to stimulate the release of DA both locally at the level of their cell bodies of origin[13,29] and distally at their projection sites[23,31,46]. Similarly, application of NT to the neostriatum was found to presynaptically augment the local release of DA[13,32].

Several lines of evidence have indicated that the interaction of NT with its receptor was followed by a ligand-induced internalization of the peptide[2,7,27,28,44]. Biochemical and autoradiographic studies in primary neurons in culture demonstrated that this internalization process was time- and temperature-dependent and that it entailed a down-regulation of the receptors on the surface of the cells[7,27,28,44]. Our own confocal microscopic studies on SF9 insect cells infected with a baculovirus encoding the NT

receptor indicated that the internalization process was endocytic in nature and involved receptor-ligand complexes[14].

Although NT receptor internalization has so far mainly been documented in epithelial and neuronal cells in culture, recent studies from our laboratory have demonstrated that this process was also operational in live brain slices[15]. Thus, using a fluorescent analogue of NT, we were able to visualize by confocal microscopy somatodendritic internalization of NT into cholinergic neurons of the rat[15] and the guinea pig[1] basal forebrain. Another line of evidence for the occurrence of NT internalization in intact brain stems comes from the work of Castel et al.[4,6] who reported retrograde axonal transport of ^{125}I-NT in the nigrostriatal pathway following its *in vivo* injection into the rat caudate putamen. This retrograde transport was deemed to be a consequence of the peptide's internalization in nigrostriatal axons as it was no longer observed when the iodinated ligand was injected together with an excess of either NT or of its bioactive fragment NT $_{8-13}$ but was unaffected by the addition of an excess of the inactive fragment NT $_{1-8}$[4]. It is still unclear, however, if this retrograde transport is selective for DA neurons as would be expected if it was indeed dependent upon receptor-induced internalization of the ligand in these cells. It is also unknown if NT is internalized, and retrogradely transported, from the dendritic arbor of these cells as they are from their axon terminals. If it is, one may ask, given that the membrane domains of dendrites and terminals exhibit differential endocytic properties[10,11,35], whether initiation from dendrites and axons gives rise to the same pattern of internalization.

To address these questions, we have examined by confocal microscopy the internalization of a fluorescent derivative of NT (fluo-NT) following superfusion of rat midbrain slices[16] and compared the distribution of the ligand to that obtained following intrastriatal injection of the same compound.

EXPERIMENTAL PROCEDURES

In vitro labeling experiments

Adult male Sprague-Dawley rats (Charles River; 280-300 g) were anesthetized with pentobarbital (65 mg/kg, i.p.) and killed by decapitation. The whole brain was rapidly removed and immersed in a cold oxygenated (95% O_2, 5% CO_2) Ringer solution containing 130 mM NaCl, 20 mM $NaHCO_3$, 1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$, 5 mM KCL, 10 mM glucose and 2.4 mM $CaCl_2$. The mesencephalon was blocked off from the mammillary bodies, rostrally, to the cerebellum, caudally, and sectioned in the coronal plane with a vibratome. Slices (350 µm-thick) were equilibrated with fresh oxygenated Ringer for 30 min at room temperature. They were then superfused with 20 nM fluo-NT at 37°C for 3 min and rinsed under a constant flow of oxygenated Ringer for 5, 10, 30 and 60 additional min. The slices were then fixed for 30 min with 4% paraformaldehyde in 0.1M PO_4 buffer, immersed overnight in a 30% sucrose solution in 0.1M PO_4 buffer and frozen at -40°C in O.C.T medium (Miles) between two glass coverslips. Incubations in the presence of 1 µM native NT or with 20 nM fluorescein isothiocyanate (Sigma, St. Louis, MO, USA) in lieu of fluo-NT were performed in parallel as negative controls.

In vivo labeling experiments

Adult male Sprague-Dawley rats were injected stereotaxically under sodium pentobarbital anesthesia (65 mg/kg, i.p) with 2 µl of the peptidase inhibitor kelatorphan (15 mg/ml, kindly provided by B.P. Roques, Paris) into the left caudate-putamen (coordinates, A:-0.3mm, L: 3.6mm, V: -6mm with respect to bregma). Ten min later, 0.5 nmols of fluo-NT were infused at three different rostrocaudal levels into the same structure (coordinates, A: 1.6, -0.8 and -3.1mm; L: 2.6, 4.1 and 5 mm; V: -5.8, -6.4 and -6.6 mm with respect to bregma). A combination of 0.5 nmols fluo-NT and 500 nmols of

native NT was injected on the contralateral side as a specificity control. The animals were killed 4.5 or 8 h later by perfusion of 4% paraformaldehyde in 0.1M phosphate buffer under pentobarbital anesthesia (65 mg/kg, i.p.). The brains were removed and post-fixed by immersion in the same solution for an additional 2.5 h. They were then cryoprotected in a 30% sucrose solution in 0.1M phosphate buffer overnight, frozen by immersion in isopentane at -50°C and stored at -80°C until used.

Tissue processing

Serial sections from fluo-NT-incubated slices and from the ventral midbrain of *in vivo*-injected rats were cut at 45 µm-thickness on a freezing microtome and collected in porcelain spot test plates containing ice-cold 0.1M phosphate buffer. Every second section was mounted on a glass slide and coverslipped with Aquamount (Polysciences, Warrington, PA, USA), for conventional epifluorescence and for confocal microscopic viewing. Alternate sections were immunostained for tyrosine hydroxylase (TH) by sequential incubation in: (1) 0.1M Tris-saline (TBS; 2 x 10 min); (2) TBS containing 0.1% Triton (1 x 10 min); (3) a 3% normal rabbit serum solution in TBS (30 min); (4) a 1/400 dilution of a rabbit polyclonal TH antibody (Incstar Corporation, Stillwater, MN, USA) in TBS containing 1% rabbit serum or an equal dilution of normal rabbit serum for specificity control (overnight at 4°C); (5) TBS (3 x 5 min); (6) a Texas red-conjugated goat anti-rabbit antibody (Jackson, Westgrove, PA, USA) diluted 1/100 in TBS (60 min); and (7) TBS (3 x 5 min). The sections were then mounted with Aquamount and analyzed by conventional epifluorescence and confocal microscopy.

Fluorescence and confocal microscopy

Sections were initially examined on a Leica epifluorescence microscope operating

with a high pressure 100-W mercury lamp and the appropriate dichroic filter combination for excitation/emission of fluorescein and Texas red.

Selected fields were further examined under a Leica confocal laser scanning microscope (CLSM) configured with a Leica diaplan inverted microscope equiped with an argon ion laser (488 nm) with an output power of 2-50mV and a VME bus MC 68020/68881 computer system coupled to an optical disc for image storage (Leica, St. Laurent, Quebec, Canada). All image generating and processing operations were performed with the Leica CLSM software package. The pictures were taken from the image monitor using a Focus Image Corder (Focus, Los Angeles, CA, USA).

Confocal microscopic images of fluo-NT labeling were acquired according to either one of the following methods depending on the degree of resolution required: (1) Twenty five serial 0.12 μm-thick optical sections were collected at 32 scans/frame and reconstituted over the whole 3 μm-thickness for high sensitivity, low resolution imaging; (2) A single optical section of 10 μm-thickness was scanned at 128 scans/frame for high resolution imaging. For all acquisitions, the gain and black levels were set manually to optimize the dynamic range of the image while ensuring that no region was completely suppressed (intensity=0) or completely saturated (intensity=255).

Double fluorescence images were simultaneously acquired in two passes, fluorescein first, Texas red second, to avoid bleeding from one channel into the other. Double-labeled polystyrene beads (diameter 10 μm; Becton Dickinson) were used to align the laser beam until both images were superimposed.

Quantitative data on the diameter of fluorescent granules, the number of fluorescent granules and the distance from each fluorescent granule to the nuclear center were obtained from optical images acquired under the second of the two imaging conditions described above. Results were expressed as the mean ±SEM of 6 measures per

experiment (n=3). Statistical analyses were performed using a one way analysis of variance (one-way ANOVA), followed by a regression curve analysis. The comparison of the slope from the regression curve was done using Student's t-test.

RESULTS

In vitro labeling experiments

The distributional pattern of fluo-NT labeling in superfused midbrain sections varied according to the duration of label washout and to the parameters of image acquisition. Five to 10 min after pulse-chase application of fluo-NT, images reconstructed from a stack of 25 individually acquired optical sections (extended focus) exhibited intense and diffuse fluorescent labeling throughout the substantia nigra, the VTA and the interfascicular nucleus (Fig 1a). In all of these regions, the labeling pervaded most of the neuropil, but appeared particularly concentrated over certain neuronal perikarya and their proximal dendrites (Fig 1a). Within the substantia nigra, the labeling was mainly evident within the SNC, but also showed finger-like extensions into the pars reticulata. Within the VTA, the labeling was highly reticular due to the sparing of the transtegmental fiber bundles (Fig 1a). The interfascicular nucleus was intensely and homogeneously labeled throughout. Thirty and 60 min after application of fluo-NT, the labeling had markedly increased over neuronal perikarya but had almost completely disappeared from the surrounding neuropil (Fig 1b).

Single optical sectioning (10 μm-thick) of the same material revealed that at all time intervals the label was mainly intracellular and in the form of small, intensely fluorescent particles (Fig 1c,d; 2a-c). However, whereas at short time intervals (5-10 min) these fluorescent particles were apparent throughout the cytoplasm of both perikarya and dendrites (Fig 1c; 2a), at longer time intervals (30-60 min) they were most heavily concentrated within neuronal perikarya (Fig 1d; 2b,c). The nuclei of fluo-NT-labeled cells remained virtually label free at all times.

Quantitative analysis of cross-sectioned nerve cell bodies showed a time-dependent decrease in the number of fluorescent particles in both the SNC [$F(1,16)=10.75$, $r=0.63$,

$p<0.01$] and the VTA [$F(1,10)=5.78$, $r=0.62$, $p<0.05$] (Fig 3; 4). Conversely, the diameter of fluorescent particles increased significantly with time, again in both the SNC [$F(1,16)=8.94$, $r=0.59$, $p<0.01$] and the VTA [$F(1,11)=11.46$, $r=0.71$, $p<0.01$] (Fig 3; 4). The evolution of these two parameters was inversely correlated with a correlation coefficient of 0.82 ($t=2.13$, $p<0.05$). The mean distance between labeled particles and the center of the nucleus decreased significantly with time in cell bodies from the SNC [$F(1,9)=9.09$, $r=0.71$, $p<0.05$] (Fig 3). The same trend was apparent within neurons of the VTA although variation in this area did not reach statistical significance [$F(1,9)=1.07$, $r=0.31$, $p>0.05$] (Fig 4). At 60 min, the fluorescent particles were virtually adjacent to the nuclear membrane as the mean distance separating them from the nuclear center was of 5 µm for the SN and 6 µm for the VTA (mean nuclear radius: SN=3.4±0.35 µm; VTA=4.85±0.35 µm) (Fig 2c; 3; 4). In slices incubated with an excess of native NT, no labeling was observed over either the SNC or the VTA except for a diffuse reactivity of intraparenchymal capillaries. Similarly, no labeling was observed after incubation with fluorescein isothiocyanate alone.

In vivo labeling experiments

Confocal laser microscopic examination of the ventral midbrain of rats injected with fluo-NT into the neostriatum revealed the presence of numerous fluorescent cells in both the SNC and the lateral part of the VTA. Serial optical sectioning of the labeled material (10 µm) showed the fluorescent labeling to be punctate and confined to the cytoplasm of nerve cell bodies (Fig 1g,h). Four and a half h after fluo-NT injection, fluorescent particles were evident throughout the cytoplasm of the cells although often predominating at one pole (Fig 1g). Eight h after injection of the ligand, the labeling was confined to the perinuclear region (Fig 1h). Quantitative analysis of labeled perikaryal

profiles indicated that the number of fluorescent particles had significantly decreased between 4.5 and 8 h, whereas the diameter of the fluorescent particles had significantly increased during the same period (Table I). The mean distance between the labeled particles and the center of the nucleus was also found to decrease with time (Table I). By 8 h, the mean distance between the labeled particles and the nuclear center (6.3±0.6 μm) was again close to the radius of the nucleus (mean nuclear radius:6.2±0.8 μm) indicating that the labeled particles were on average closely apposed to the nuclear membrane.

Codistribution of fluo-NT labeling and TH immunoreativity

Simultaneous acquisition of fluo-NT (green) and TH immunofluorescence (red) in double-labeled preparations revealed a close regional overlap between fluo-NT labeling and TH-immunoreactivity throughout the SNC and the VTA. At the cellular level, the vast majority of fluo-NT-labeled cells in both regions were found to be TH-immnunopositive following either local superfusion with or intrastriatal injection of the fluorescent probe (Fig 1e, f).

DISCUSSION

Previous studies carried out on neuronal cells in culture[17,18,27,28] as well as on slices of rat[15] and guinea pig[1] basal forebrain have shown that NT may be internalized throughout the somatodendritic domain of its target cells as a consequence of its interaction with specific cell surface receptors (for a review, see Beaudet et al.[2]). Furthermore, *in vivo* studies on the rat nigrostriatal system have implicated a similar process at the level of axon terminals for the initiation of the retrograde axonal transport observed following intrastriatal injection of ^{125}I-NT[4].

In the present study, we have resorted to a fluorescent derivative of NT, fluo-NT, to investigate NT internalization from the somatodendritic domain of nigrostriatal and neocorticolimbic DA cells and to compare this internalization to that observed following intrastriatal administration of the peptide. Fluo-NT was previously shown by us to bind with the same selectivity and affinity as native NT to central NT receptors and to provide for sensitive confocal microscopic detection of NT endocytosis in both cell culture preparations and rat brain slices[16,17].

Following its application onto rat midbrain slices, fluo-NT was indeed found to be internalized throughout the perikarya and dendrites of ventral tegmental neurons. Experiments in which fluo-NT labeling was combined with TH immunostaining confirmed that this internalization was selective for DA cells, i.e. for cells selectively endowed with NT receptors in this region of the brain[42]. This finding, together with the fact that the labeling was totally abolished when carried out in the presence of an excess of native NT, is consistent with the receptor-dependence of the internalization process previously demonstrated *in vitro*[17]. High resolution confocal microscopic scanning indicated that fluo-NT was internalized through small vesicular compartments the size and shape of

which were compatible with those of endosomes[35]. This observation is congruent with the results of earlier biochemical[7,28] and morphological studies[15,17] on neural cell lines which have shown the internalization process to be endocytic in nature.

The concomitant decrease in neuropil labeling and increase in perikaryal fluorescence observed in time course experiments strongly suggest that internalization occuring at the level of the dendritic tree is rapidly followed by an intracellular mobilization of internalized molecules towards the perikaryon of the cells. The parallel reduction in number and increase in size of intracellular fluorescent particles observed during this migration further suggests that the internalization compartments fuse en route towards the centre of the cell, as they would be expected to do if they were to give rise to multi-vesicular bodies and, eventually, to lysosomes, i.e. if the internalization proceeded along classical endocytic pathways[20,22,43,45]. This interpretation is in keeping with the time-dependent reduction in the mean distance separating fluorescent particles from the nuclear center as lysosomes have been shown to be mainly concentrated within the perinuclear region[19].

Our results following *in vivo* injection of fluo-NT into the neostriatum confirmed that NT molecules internalized at the level of axon terminals were retrogradely transported towards their nerve cells bodies in the SN and the VTA. They also demonstrated, through the use of concurrent TH-immunostaining, that this retrograde labeling was selective for DA neurons, a finding congruent with an initiation of the transport through a NT receptor-dependent endocytic process. Within retrogradely labeled perikarya, internalized fluorescent ligand molecules (and/or their metabolites) were concentrated within small, round particles similar in size and distribution to the ones detected in the same regions following local superfusion with fluo-NT. The evolution with time of these intracytoplasmic fluorescent granules was also similar to that observed following

somatodendritic internalization of the ligand. Thus, whereas 4.5 h after fluo-NT administration the fluorescent particles were small, numerous and distributed throughout the cytoplasm, after 8 h, they were large, sparse and clustered against the nucleus. These data confirm and extend the results of earlier autoradiographic studies on the ultrastructural distribution of retrogradely transported radioactivity following *in vivo* administration of ^{125}I-NT[6]. In this earlier study, a significant proportion of intracytoplasmic silver grains was found to be associated with organelles such as clear vesicles, multivesicular bodies, and lysosomes[2,6] which likely correspond to the fluorescent particles vizualized in the present experiments. However, a large proportion of silver grains showed no obvious association with vesicular elements, which is at odds with our confocal microscopic observations. This discrepancy is most likely attributable to differences in the fixation protocols between the two studies. Indeed, autoradiographic studies were carried out on tissue fixed with glutaraldehyde, a cross-linking agent likely to retain both vesicular and extravesicular molecules of NT and/or NT degradation products[36]. In contrast, confocal microscopic experiments were performed after fixation with paraformaldehyde, a fixative much less prone than glutaraldehyde to retain non-vesicular NT and/or its metabolites.

Taken together, the present results indicate that fluo-NT is internalized in nigro-striatal DA neurons from both their somatodendritic and terminal domains and that it is subsequently transported centripetally to the nerve cell body. This intracellular migration appears to be carried out through classical endocytic pathways, although double labeling and/or further electron microscopic studies are clearly needed to confirm the identity of the observed sequestration compartments.

Irrespective of their origin, retrogradely transported "endosomes" were found to coalesce with time and eventually cluster in the perinuclear area. Surprisingly, there was

no obvious quenching of the internalized fluorescent product in these late (i.e. presumably lysosomal) compartments as would have been expected had they been acidic[24,40]. Since acidification of the endosomal system reportedly accounts for dissociation of receptor ligand complexes[9], one may surmise from this observation that the fluorescent molecules carried up to the perinuclear zone mainly correspond to molecules of ligand non-dissociated from their receptors. This interpretation would be in keeping with the lack of recycling of internalized NT receptor molecules[7]. It would also conform to the results of biochemical studies which have shown that up to 50% of the radioactivity detectable in nigral neurons 2.5 h after intrastriatal injection of ^{125}I-NT corresponds to unmetabolized NT[5]. Why internalized NT molecules would thus be protected from metabolic degradation is still a matter of debate. Laduron and co-workers have postulated that internalized ligand molecules, their receptors and/or fragments thereof might be translocated to the nucleus and thereby regulate gene expression within their target cells (for a review, see Laduron et al.[25]). Reports of specific nuclear binding sites for NT[8] as well as for other neuropeptides such as VIP[33] and somatostatin[26] support this hypothesis. Furthermore, injection of NT into the neostriatum was shown to be accompanied by an increase in TH mRNA within nigral neurons according to a time course consistent with the arrival of retrogradely transported ligand molecules[3]. Pending further evidence for its involvement in genomic regulation, however, one cannot exclude the currently accepted view that the internalization process mainly subserves the clearance of ligand molecules and the concomitant down-regulation of their receptors.

In any event, the present results strongly support a physiological role for neuropeptide receptor internalization in mammalian brain and demonstrate that this biological process may be harnessed for the selective identification of neurons endowed with a specific type of neuropeptide receptors.

FIGURES

Figure 1.

Confocal microscopic images of fluo-NT labeling in the rat mesencephalon. Images in (a), (b), (e) and (f) are reconstructed from a stack of 25 serial 0.12 μm-thick optical sections scanned at 32 scans/frame. Images in (c), (d), (g) and (h) are single 10 μm-thick optical sections scanned at high resolution (128 scans/frame).

a-d: Labeling in the VTA following local superfusion with 10 nM fluo-NT. Sections scanned 10 (a), 30 (b,c) and 60 (d) min after fluo-NT washout. At 10 min (a), labeling is evident over both perikarya (arrows) and neuropil. At 30 min (b), nerve cell bodies are still intensely labeled, but the neuropil labeling is markedly reduced. High resolution scanning (c, d) shows labeling in the form of small punctuate fluorescent granules that pervade the cytoplasm of perikarya (arrows) and dendritic processes (arrowheads). Note that at 60 min (d), the fluorescent particles are larger and more predominantly perikaryal than at 30 min (c).

e-f: Brain mesencephalic neurons dually labeled for fluo-NT (e) and tyrosine hydroxylase (TH) (f) following 3 min superfusion with fluo-NT. Images were acquired using two different channels to avoid overlap of fluorophore emission spectra. Note the presence in (e), but not (f) of endosome-like particles (arrows).

g-h: Midbrain sections scanned 4.5 h (g) and 8 h (h) after intrastriatal injection of fluo-NT. Note that in (g) the fluorescent particles are small and distributed throughout the perikaryon, whereas in (h) they are large and located in the perinuclear region. Scale bars: 10 μm.

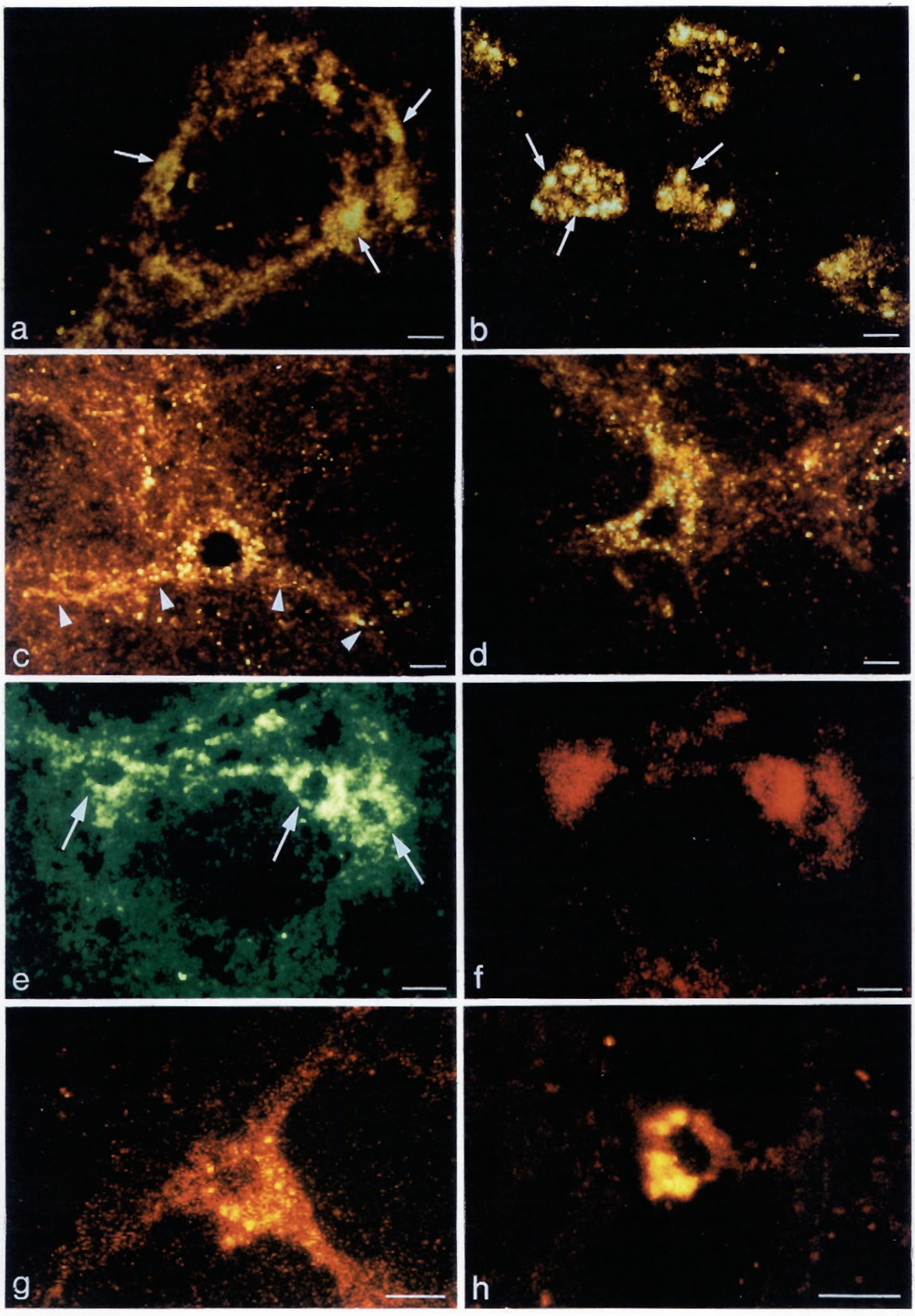

a
b
c
d
e
f
g
h

Figure 2.

High magnification confocal microscopic images of fluo-NT labeling in slices of the rat VTA. Sections scanned 5 (a), 30 (b) and 60 (c) min after local application of fluo-NT. Images correspond to 10 µm-thick optical sections scanned at high resolution (128 scans/frame).

a: At 5 min, the label is detected within small endosome-like structures dispersed throughout the cytoplasm of the cell (arrows).

b: At 30 min, the granules are larger than at 5 min (arrows) and begin to cluster in the nuclear region. Note that fluorescent particles are still apparent in proximal dendrites at this time (arrowheads).

c: At 60 min, fluorescent granules are all large and in the immediate vicinity of the nucleus (N). Scale bars: 10 µm.

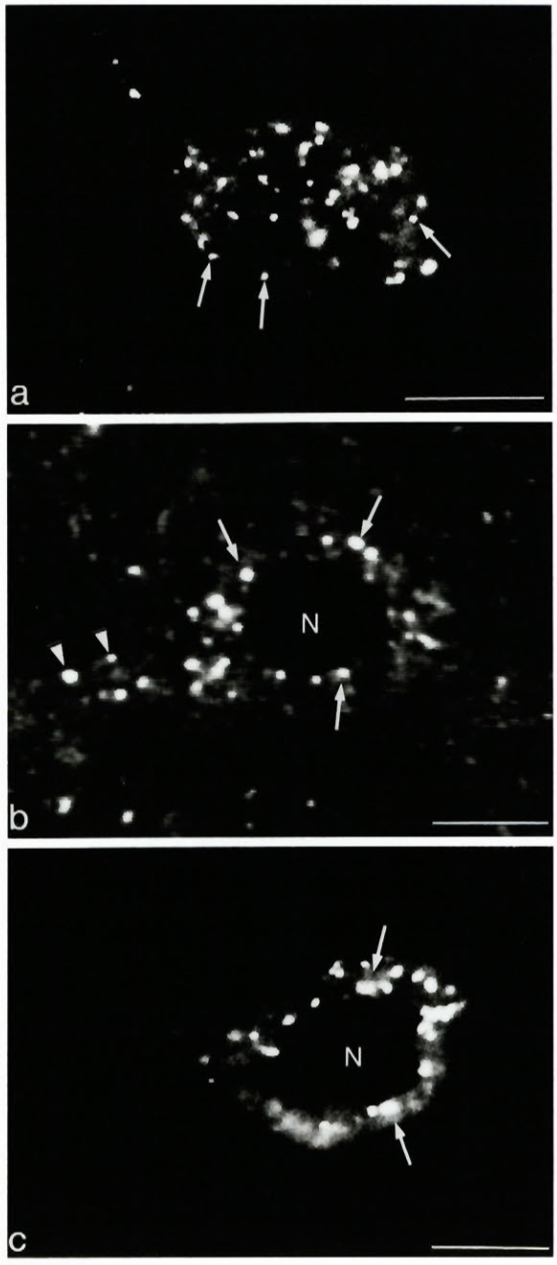

a
b
N
c
N

Figure 3.

Evolution over time of the mean number (A), diameter (B) and distance to the nuclear center (C) of fluorescent endosome-like particles in 10 µm-thick cross-sections of fluo-NT-labeled perikarya in the SNC. Slices were superfused with fluo-NT during 3 min and rinsed out for 5-60 min. Mean ± SEM of 12 slices from 4 animals.

1

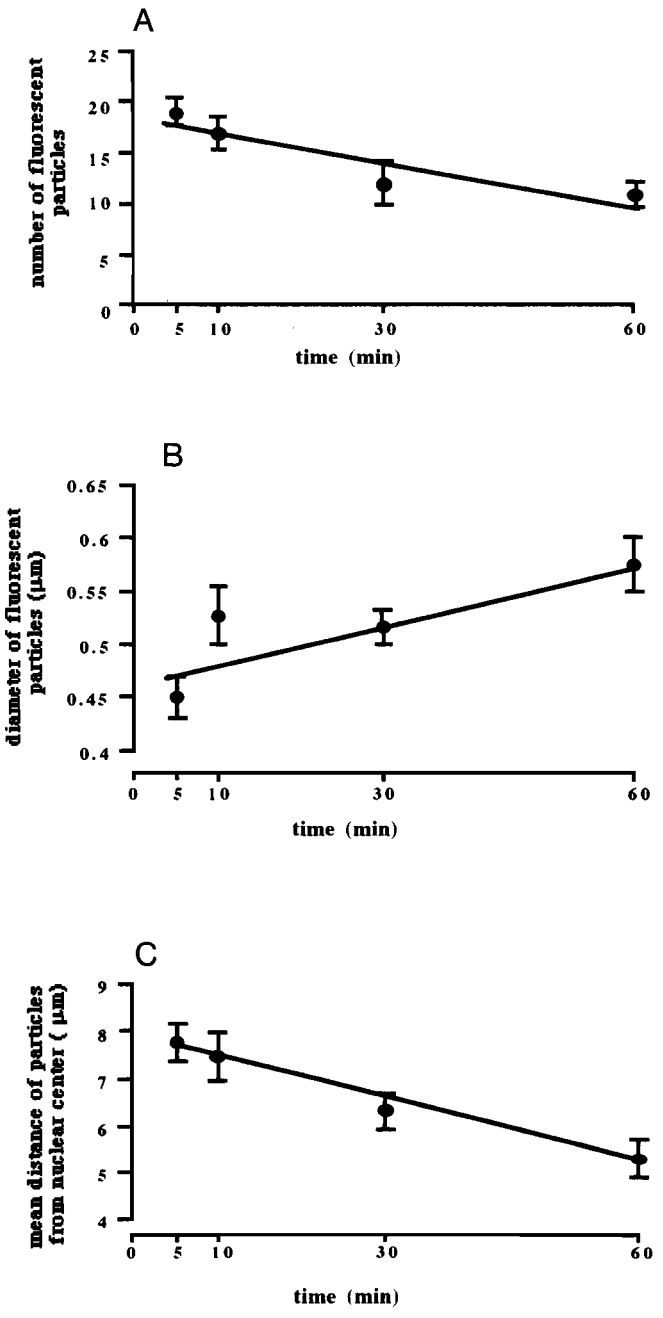
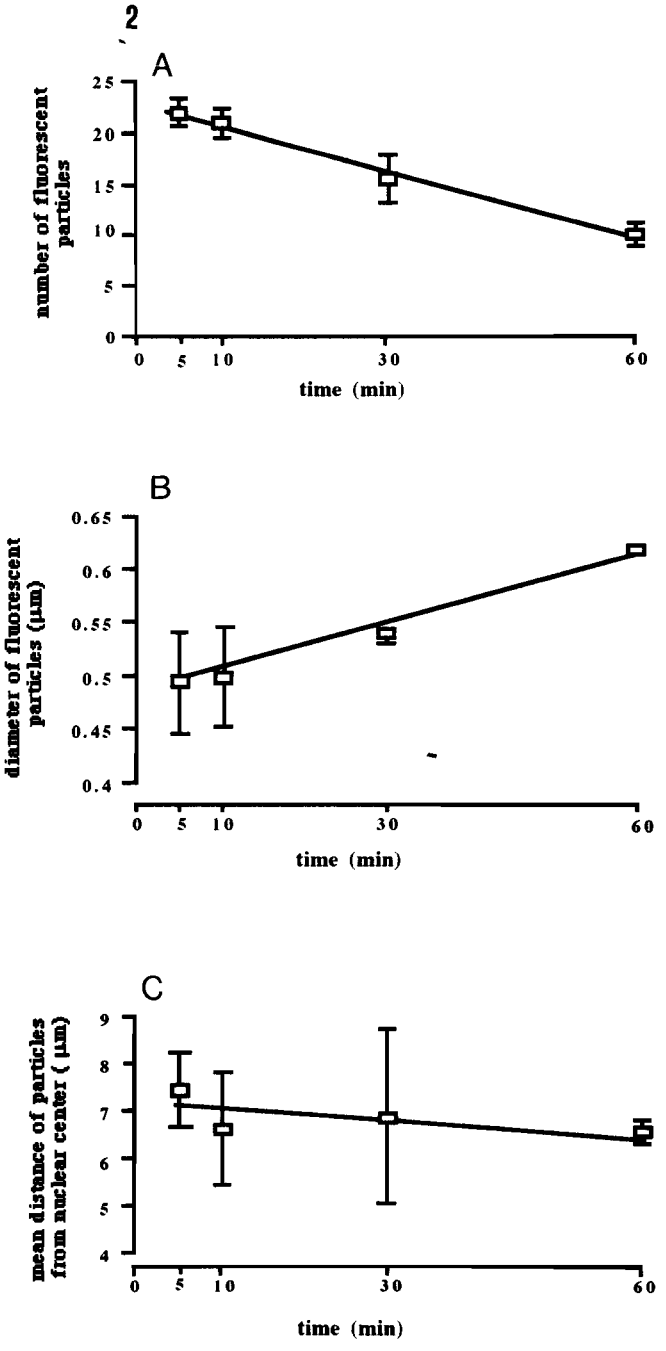

A
number of fluorescent particles
25
20
15
10
5
0
0
5
10
30
60
time (min)
B
diameter of fluorescent particles (µm)
0.65
0.6
0.55
0.5
0.45
0.4
0
5
10
30
60
time (min)
C
mean distance of particles from nuclear center (µm)
9
8
7
6
5
4
0
5
10
30
60
time (min)

Figure 4.

Evolution over time of the mean number (A), diameter (B) and distance to the nuclear center (C) of fluorescent endosome-like particles in 10 µm-thick cross-sections of fluo-NT-labeled perikarya in the VTA. Slices were superfused with fluo-NT during 3 min and rinsed out for 5-60 min. Mean ± SEM of 12 slices from 4 animals.


A
number of fluorescent particles
25
20
15
10
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0
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5
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30
60
time (min)
B
diameter of fluorescent particles (µm)
0.65
0.6
0.55
0.5
0.45
0.4
0
5
10
30
60
time (min)
C
mean distance of particles from nuclear center (µm)
9
8
7
6
5
4
0
5
10
30
60
time (min)

Table I

Morphometric analysis of fluorescent particles in neurons of the ventral midbrain following intrastriatal injection of fluo-NT*

	4.5 hours	8 hours
diameter of particles (μm)	2.05 ± 0.14	3.83 ± 0.93
number of particles	7.00 ± 1.00	4.00 ± 0.50
distance to nuclear center (μm)	8.60 ± 0.90	6.30 ± 0.60

*Mean ± SEM of 6 measures per experiment (n=3)

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CHAPTER 6: GENERAL DISCUSSION

In order to elucidate the mechanisms of action of neuropeptides in the central nervous system, the receptors for neurotensin, a tridecapeptide whose role as a neuromediator is established, were visualized by confocal microscopy.

One of the main contributions of this thesis is the synthesis and characterization of a new fluorescent probe for neurotensin receptors (NT-R). This novel tool was instrumental in our gaining knowledge concerning the internalization of neurotensin (NT) in targeted neurons. Fluo-NT, a derivative of the peptide labeled with fluorescein, binds with high affinity to SF9 cells infected with a baculovirus which codes for the neurotensin receptor and to hybrid neuron-neuroblastomas derived from mouse septum. This fluorescent ligand was also shown to bind to living neurons in basal forebrain and mesencephalic tegmentum slices which are identified immunocytochemically as being cholinergic and dopaminergic, respectively.

The present thesis provides the first demonstration of receptor-induced internalization of a neuropeptide (NT) in live brain slices. This internalization is time- and temperature-dependent and proceeds from both the entire somatodendritic arbour of the cells and axon terminal plasma membranes through endosomal-like structures which are retrogradely transported towards the cell center.

The NT/NT-R internalization and transport processes demonstrated in the present work thus initiate or participate in slow and complex intracellular regulatory processes including the termination peptidic signal, the down-regulation of cell surface receptors, and finally the regulation of mRNA expression.

The ligand

The association of NT-R with neuronal cholinergic and dopaminergic subpopulations suggested to us that specific NT-R ligands might be used as selective markers for the identification of neurotransmitter-specific target cells in the CNS. This thesis demonstrates that NT may be labelled at its amino terminus with a fluorophore without significant deleterious effects on the binding of the neuropeptide to its receptor.

Epifluorescence and confocal microscopic analysis of specific fluo-NT binding to sections of the rat midbrain revealed a topographic distribution of the bound fluorescent ligand that was similar to that previously observed with autoradiography using radiolabeled NT. However, fluo-NT provided a markedly higher cellular resolution than did the radiolabeled ligand and enabled, in particular, the detection of hitherto unnoticed intracytoplasmic receptor clusters. Confocal microscopy's chief advantage for the present work is the ability to generate non-invasive images of optical sections of labelled cells with a virtual absence of out-of-focus blur, which may subsequently be digitally enhanced, morphometrically analyzed and photographically recorded. Furthermore, confocal microscopy allows us topographical and morphological studies of the specific fluorescent labeling in intact brain slices. The possibility of double or triple labelling any neuronal population which possesses NT-R by combining fluo-NT binding and/or internalization with simple or double immunocytochemistry opens new avenues for our understanding of the chemical neuroanatomy of complex systems utilizing CLSM. The double-labelling approach developed in this thesis is applicable to other brain regions, other peptide receptors with new fluorescent peptides (Beaudet et al., preliminary observations), and various types of cells, allowing for the precise determination of target cells expressing a given receptor. Due to the complexity of brain circuitry, this kind of approach could improve our understanding of the interaction between neuromediators and the physiological meaning of these interactions in brain function or pathology.

Another novel application of fluo-NT concerns its use in flow cytometry. Flow-

cytometry is a technique currently used with fluorescent labelled antibodies and has been proven to be useful for the identification and analysis of antigens present on the cell surface. Since antibodies to peptide receptors are scarce, fluo-NT may be utilized to provide information on NT-R pharmacology and the characteristics of NT-R bearing cells (i.e. size, granularity, etc...). For example, since NT-R are selectively associated with cholinergic neurons in the basal forebrain and DAergic neurons in the mesencephalon, these may be selectively isolated from tissue from either of these two areas by flow cytometry using fluo-NT as a cell surface marker. Once sorted, this purified cell population can be further studied.

The following discussion will examine, from a spatio-temporal point of view, the cellular evidence, both anatomical and functional, for the internalization and translocation of NT in targeted neurons.

Binding of fluo-NT to neuronal cells

Cell surface fluo-NT/NT-R binding was examinated at 4°C on cholinergic hybrid cells. After one hour incubation at this temperature, microaggregates and clusters of NT receptors were observed on the cell surface. It has been previously shown in other cell types that receptor clustering is accompanied by a conformational perturbation of the receptor which results in cell activation (Velazquez et al., 1989; Jan et al., 1990; Hollenberg et al., 1991). The initial signal generated by a ligand-activated receptor must be greatly amplified to yield an overall cellular response. This signal may be amplified via the generation of numerous intracellular second messenger molecules and/or via the opening of specific channels. In a collaborative work, we demonstrated that in cholinergic neurons, bath application of fluo-NT induces a membrane depolarization associated with a decrease in input conductance (Alonso et al. 1992; Alonso et al., 1994). Most significantly, fluo-NT stimulation led to the emergence of a very prominent slow rhythmic bursting pattern that often developed into complex spindle-like sequences. These results

demonstrate for the first time the electrophysiological functionality of NT-R on basal forebrain neurons and the existence of a receptor-mediated internalization of NT in these cells.

Internalization of neurotensin receptor

Although mainly documented for large polypeptides acting through receptor tyrosine kinases, receptor mediated internalization has also been reported for a number of smaller peptides acting through G-protein-coupled receptors. For instance, biochemical and/or histochemical studies have provided evidence for receptor-induced internalization of somatostatin (Morel et al., 10985), as well as of gonadotropin (Hazum 1980 et al.; Naor et al., 1981; Wynn et al ., 1986; Morel et al., 1987), corticotrophin (Leroux et al., 1984) and thyrotropin releasing hormones (Morel et al.) in cells of the anterior pituitary. There have also been reports on the internalization of vasoactive intestinal peptide in lymphocytes, acinar pancreatic cells (Anteunis et al., 1989) and intestinal epithelial cells (Izzo et al.,1991); of vasopressin internalization in kidney and smooth muscle cells (Lutz et al., 1990), and of atrial natriuretic factor in heart muscle. Our work on NT has demonstrated that this process may be operational in CNS neurons.

In all our cellular models, the binding of neurotensin induces a rapid internalization of the receptor. Both biochemical (acid washes) and anatomical (serial optical sectioning of labelled cholinergic hybrid cell) experiments support the concept of time- and temperature-dependent entry of NT inside the neuron. The internalization is blocked by both phenylarsine oxide, an endocytosis inhibitor and by lowering the temperature between 0°C and 4°C. That this internalization is receptor-mediated is supported by the fact that it is abolished in the presence of non-fluorescent NT, that its kinetics are similar to those of NT binding to cell surface receptors (cholinergic hybrid cells), and that it is selective toward neurons documented to harbour high affinity NT-R such as basal

forebrain cholinergic neurons or midbrain DAergic neurons. Moreover, in SF9 insect cells transfected with cDNA coding for high affinity NT-R and cholinergic hybrid cell cultures, we have shown that following covalent cross-linking of a nitro-azido derivative of fluo-NT to cell surface receptors, internalization proceeds in the same fashion as without cross-linking, confirming that in the case of NT, it is the receptor-ligand complex which is transported through the cell.

After NT binding to cell surface NT-R, the ligand receptor complexes may accumulate in coated pits , bud from the early endosomes which then may fuse or be delivered to larger late endosomes. To address this question of whether the internalized complexes are transported in the cytoplasm by clathrin-coated pits, one could detect the internalized fluo-NT using an antibody against fluorescein. This antibody may be subsequently visualized coupled with gold in order to determine, by electron microscopy, the precise subcellular nature (coated pits or not) and localization of the internalized material.

Dendritic and axonal unidirectional transport

Past studies have largely concentrated on endocytosis from nerve terminals as a response to neurotransmitter release or as a prelude to retrograde transport towards the cell body. Here, we have studied the neuron as whole in term of traffic from both the dendritic and axonal domains back to the cell body.

On the one hand, confocal microscopic analysis of basal forebrain and mesencephalic tegmentum *ex vivo* preparations incubated at 37°C, illustrates that the intracellular distribution of the fluorescent ligand reorganizes with time. At short time intervals, the labeling is distributed throughout the cytoplasm of neuronal cell bodies and their dendrites. With time, the intensity and the size of granules increase in the cell bodies but diminish in the neuropil, suggesting a dendro-somatic centripital transport of the ligand-receptor complex. This transport involves the binding of NT to its receptors

located on dendrites and perikaria followed by internalization of receptor-ligand complexes.

On the other hand, confocal microscopic examination of the ventral midbrain following intrastriatal injection of fluo-NT *in vivo* confirmed the existence of a retrograde transport of the internalized tracer in dopaminergic neurons. Overall, our studies have shown that fluo-NT is rapidly internalized within numerous, small, granular structures located in the neuron periphery. With time, these vesicles become larger and less numerous and become mainly concentrated in the perinuclear area. The similarity in size and morphology of the retrogradely transported material from the somatodendritic and nerve terminals leads us to speculate that this material is unidirectionaly retrogradly transported back to the cell body. Retrograde axonal transport from the synapse to the perikaria has been clearly demonstrated for NGF, opiates, and NT (Hendry et al.,1974; Laduron et al., 1987; Castel et al., 1990). Internalization of the ligand-receptor complex is a prerequiste for retrograde transport.

It has been shown in the case of NT-NTR that retrograde transport is inhibited by colchicine and therefore microtubule-dependent (Castel et al., 1992). The nature of microtubules associated proteins is different in dendrites and axons belonging to the microtubule-associated protein and tau peptide family, respectively (Bass et al., 1988). One may ask whether the mechanisms of transport of internalized material are similar when initiated from dendrites or axon terminals. It would be interesting to immunohistochemically label microtobules, actin or other cytokeletal elements, in combination with fluo-NT in order to determine which ones are involved and during what portion of the internalization pathway.

Endosome-like-structure

The intracellular migration of endocytic organelles is reminicent of that observed in neurons in culture for markers of the constitutive endosomal system. The question arises

as to whether these fluorescently-labelled granules truly correspond to endosomes? One way of answering this question would be to use different antibodies to the rab G-protein family, which are known to specifically label different endosome types, in combination with any of the models described in the present thesis following fluo-NT internalization. In fact, it has been proposed that various rab proteins (over 30 identified to date) may shuttle back and forth from specific donor and acceptor compartments (Simons et al., 1993). Further work is necessary to find out whether the late endocytic compartments in the neuronal cell body receive endocytosed material from both the axon and the dendrites or wether the late endocytotic compartments are separated into axonal and dendritic subclasses. Identifying their distribution with retrogradely labeled compartments of fluo-NT sequestration would therefore provide essential information on intracellular sorting machinery.

Neurotensin/Neurotensin receptor internalization and transport functions.

The internalization of peptide-receptor complexes can subserve a variety of cellular functions, through as yet unknown mechanisms. The high rate at which the NT internalization process occurs is in agreement with one of its functions might be the removal/inactivation of bound NT. This could conceivably be a mechanism for ensuring that once bound to receptor, the ligand cannot dissociate and bind to another receptor. Internalization could be a mechanism for clearing ligand from the extracelluar space. In short, one can see this process as contributing to the termination of ligand action. The presence of endopeptidase 24.16, an enzyme shown to play a major role in the functional inactivation of NT (Chabry et al., 1990), inside NT target cells (Woulfe et al., 1992) is consistent with this interpretation. Further work is necessary to determine whether the internalized fluo-NT is co-localized with this enzyme, and in which specific subcellular compartments (i.e in early endosomes).

A second possible role for internalization is that of receptor regulation (Collins et

al., 1992). Several mechanisms have been evoked to explain the desensitization of receptors. Molecular desensitization is due to agonist-induced conformational changes as is mainly the case for all multimeric ligand-gated ion channels. Cellular desensitization of G-proteins-coupled receptors as well as receptors not coupled to G-proteins occurs through processes involving agonist-induced receptor internalization, uncoupling, phosphorylation and down-regulation. Receptor regulation by internalization has been demonstrated for a wide variety of receptors of neuropeptides, cytokines, growth factors and classical neurotransmitters. It has been shown that internalization of receptors in cells was followed by receptor recycling and/or degradation after fusion of internalized vesicles with lysosomes. Prolonged incubation of agonist with NT-R endowed cells has been shown to result in a decrease in the total cell surface receptor number (Vanisberg et al., 1991; Yamada et al., 1993; Donato Di Paola et al., 1993; Hermans et al., 1994).

As previously suggested by others on N1E-115, HT29 cells and in neuronal primary cultures (Vanisberg et al.,1991; Tuner et al., 1987; Mazella et al., 1991), NT-induced receptor internalization results in a down-regulation of the receptor, thereby providing an adaptive change to neurotensinceptive neurons in response to high extracellular concentration of NT. However, this is not the only mechanism to regenerate receptor sensitivity. Recently, Mazella et al (Mazella et al., 1993) have proposed the existence of spare receptors which appear on the cell surface after initial NT-R internalization. This phenomenon is in agreement with Vanisberg (Vanisberg et al.,1991) which suggested that the NT-R does not appear to be recycled back to the cell surface after internalization.

A third possible function for internalization is its key role in intracellular signalling which does not involve degradation or recycling of receptor/receptor-ligand complexes, and which involves the retrograde transport of these internalized complexes toward the cell body (Laduron, 1994).

Membrane receptors (NT-R) have to convey a signal from the exterior to the

interior of the cell since the ligand (NT) does not cross the cell membrane and thereby provides greater selectivity for its receptor. Membrane receptors (NT-R) would therefore exert a double function in different cell compartments: first, a short-term effect and then a long-term response. These effects appear to be mediated via different systems (Laduron, 1994). Short terms effects occur through the transduction mechanism, the second messengers and ion channels in the vicinity of membrane receptors, as has been shown for NT which increases DA release through presynaptic receptors located on DAergic nerve terminals. In long-term responses, the NT-receptor complex may serve as a signalling molecule, which carries information (cytoplasmic or axonal transport of internalized vesicles) to the cell body from a distant domain. NT in subtantia nigra has been shown to increase the synthesis of tyrosine hydroxylase of DAergic neurons after retrograde axonal transport of the ligand-receptor complexes (Castel et al., 1994).

The idea that internalization of peptide-bound receptors is necessary for the induction of their long-term effects is an emerging concept. However, many other systems have already been shown to require ligand-mediated receptor internalization to elicit long-term effects. The cytokines Il-1 and IL-2, for example, induce nuclear translocation of membrane receptors which are directly involved in changes in gene expression (Grenfell et al, 1991; Jothy et al., 1989). Several growth factors (nerve growth factor (NGF), fibroblast growth factor) interact with specific membrane receptors and behave as mitogens or agents affecting the development and maintenance of targeted populations of cells and neurons. One of the main features of NGF is that it can be internalized at nerve terminals and then retrogradely transported to the cell body and nucleus (Rakowicz-Szulczynska et al., 1986). Other peptidic ligands, such as insulin, prolactin, epidermal growth factor have also been found, after internalization, to be translocated to the nucleus (Soler et al., 1989 ; Rakowicz-Szulczynska et al., 1986).

For the neuropeptides, evidence of nuclear receptors is scarce and often indirect. For NT, we showed that NT-receptor complexes are translocated to the perinuclear

region of targeted neurons. The presence of granular fluorescence in the vicinity of the nuclei and the absence of nuclear labeling suggests to us that fluo-NT is not cleaved until the complexes reach its site of action; otherwise fluorescent amino acid fragments resulting from the degradation of NT would have been washed out during the technical processes (fixation and washing). We may assume that once internalized, the NT-receptor complexes travel in endocytotic vesicles, where they are protected from enzymes until they reach the cell center. This observation is in agreement with Castel (Castel et al.,1992) which showed that after intrastriatal injection of radiolabeled-NT and axonal retrograde transport in Daergic neurons, the cytoplasm still contained 50% of undegraded NT (Castel et al., 1994). Therefore, one may hypothetize that NT-R transport in targeted neurons occurs with NT bound to its receptor in endosome-like structures. The latter may fuse with the nuclear membrane and dissociation of the complex may then occur through the intracytoplasmic action of endopeptidase 24.16 (Woulfe et al., 1992) where upon the NTR itself may be released and available to be translocated to the nucleus.

The question as to how signals originating from NT receptor internalization and intracellular transport affect gene expression (long-term effects) remains. It has been suggested that due to homologies between certain domains of the NT receptor and specific DNA binding proteins (Laduron et al., 1992; Hermans et al.; 1994), NT-R or a segment thereof, following internalization, may interact with chromatin. However, the receptor itself and/or the receptor-ligand complex may interact with DNA following internalization. Since receptors not coupled to G-proteins also internalize through a ligand-induced process, it is not necessary that the heterotrimeric G-protein serve as triggering signals. For the most part, specific receptor domains involved in regulating gene expression have yet to be determined. NT-R itself may also trigger an intrinsic kinase activity via phosphorylation-dephosphorylation reactions. The latter may act on secondary enzymes which could themselves interact with the nucleus. In summary, we may conclude that the internalization processes observed herein were confined to neurons

endowed with high affinity receptors, occurred over the entire neuronal surface, was linked to receptor activation, involved the receptor ligand complex and seemed to borrow classical endocytotic pathways. That this process may be a general phenomenom occurring with other peptides acting on other targeted neuronal systems. A fluorescent derivative of somatostatin has been shown to be internalized in cells of the arcuate nucleus, via an internalization pathway similar to that for neurotensin.

CHAPTER 8: SUMMARY

The results contained within this thesis may be summarized as follows:

1) Fluo-NT is a selective marker for NT receptors and exhibits pharmacological and biological activities similar to those of native NT.

2) Fluo-NT selectively binds to, and internalizes within, hybrid cholinergic cells in a time- and temperature-dependent fashion.

3) This internalization is ligand-induced (i.e. is not constitutive) and is receptor-mediated.

4) The process of internalization involves the entire ligand-receptor complex.

5) *ex vivo* superfusion of rat basal forebrain slices with fluo-NT results in the binding and internalization of the fluorescent compound in a selective population of neurons activated by NT and immunohistochemically identified as cholinergic neurons.

6) NT is internalized at the level of both the perikaria and processes of basal forebrain neurons through small endocytotic compartments the size and the shape of which are compatible with those of endosomes.

7) The internalization of neurotensin occurs both at somatodendritic and axon terminal plasma membranes in dopaminergic neurons.

8) The pattern of labeling induced by fluo-NT internalization is consistent with a retrograde transport of endosome-like organelles.

9) The internalization process is rapidly followed by an intracellular mobilization of these endosome-like particles from dendrites and from the periphery of the cell to its perinuclear region.

10) The concomitant reduction in number and increase in size of fluorescent particles further suggests that the internalization compartments coaless and/or fuse en route towards the center of the cell, as they would be expected to do if they were to give rise to multi-vesicular body and, eventually, to lysosomes.

11) These results indicate that the internalization process itself may be used for selective identification of NT target cells in live brain.

APPENDIX

INTERNALIZATION AND INTRACELLULAR MOBILIZATION OF NEUROTENSIN IN NEURONAL CELLS

Alain Beaudet, Jean Mazella[1], Dominique Nouel, Marie-Noëlle Castel*, Pierre Laduron[2], Patrick Kitabgi[1], and Marie-Pierre Faure

Laboratory of neuroanatomy, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec, Canada H3A 2B4
[1]CNRS, UPR 0411, Université de Nice-Sophia Antipolis, 06560 Valbonne, France
[2]Laboratoire de Neurochimie et Service de Neurobiologie, Université Catholique de Louvain, 10 avenue Hippocrate, 1220 Bruxelles, Belgium.
*** Present address: Laboratoire de Génétique Moléculaire de la neurotransmission et des processus neurodégéneratifs du CNRS, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France.**

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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 [1],[2],[3]). In neurons, 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],[7],[8], beta 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 mu 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 intra-cerebral injection in 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]. By contrast, other neuropeptide receptors, such as those for cholecystokinin, do not 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 with 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 intra-cellular 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*.

Ligands

The experiments described herein were carried out using either monoiodo neurotensin (^{125}I-NT) or N_ fluoresceinyl-thiocarbamyl (FTC)-[Glu^{1}] neurotensin (FTC-NT; fluo-NT). The iodinated ligand was prepared by a lactoperoxidase H_2O_2 method and purified (spec.act.2000Ci/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 high pressure liquid chromatography [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].

STUDIES IN NEURONAL CELL CULTURES

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. Hamond and N.R. Cashman [28]. 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 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°C or 37°C with 0.2 nM ^{125}I-NT in Earle's Hepes buffer, in the presence of peptidase inhibitors, and with or without a hundred 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°C and 37°C, 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°C than at 4°C in both preparations. Furthermore, whereas the maximal binding capacity (B_{max}) at 4°C was comparable to that observed on membrane homogenates prepared from the same type of cells, the one observed at 37°C was consistently higher, suggesting that the B_{max} at 4°C corresponded to the original number of cell surface receptors whereas the one at 37°C reflected a temperature-dependant 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 attributable to receptor internalization. First, when ^{125}I-NT was incubated with neurons at 37°C but in the presence of 10 μM of the endocytosis inhibitor, phenylarsine oxide, the maximal specific binding was similar to that seen at 4°C, (i.e. 160 fm/mg prot; [32],[34]). Second, when cells incubated at 37°C 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-dependant.

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°C or 10°C [13]. In these experiments, 10°C was preferred to 4°C as it provided for better morphological preservation without significantly increasing internalization, at least within the time frame of our experiments. At all time intervals examined (5,30,60 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°C, the proportion of grains associated with neuronal perikarya (as opposed to

neuronal processes) was much greater at 37°C than at 10°C (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°C (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°C 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°C and 37°C 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, a 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°C 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°C revealed the label to be confined to the cell surface (Fig.5a). The absence of intracellular staining was particulary 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°C after saturation of cell surface receptors, the labeling 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°C 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 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 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 h, 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 in the introduction, *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 a selective retrograde labeling of neurons in the substantia 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 IP) with 2 ml of kelatorphan (15 mg/ml) followed 10 min later by 0.5 nmols of fluo-NT into the left caudoputamen. The animals were killed 4.5 or 8 h later by perfusion with 4% parafamaldehyde and the brains cut on a freezing microtome after overnight cryoprotection in 30% sucrose. Sections of the midbrain were examined by confocal microscopy before or after immunohistochemical counterstaining with an antibody against tyrosine hydroxylase.

At both times studied, confocal microscopic examination of 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 h 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 h, 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 h, 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 suggest that once they have reached the perikaryon, these sequestration compartments coaless in the perinuclear zone.

Electron microscopic studies

To further elucidate the subcellular compartmentation of the retrogradely transported ligand, electron microscopic autoradiography was carried out on sections of the midbrain 4.5 h after injection of ^{125}I-NT in 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 analyzed by direct scoring, i.e. by identifying the underlying structure without correcting for possible cross-fire, a significant proportion of the grains was 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 discrepancies 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 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 h after intrastriatal injection of ^{125}I-NT still corresponds to the unmetabolized peptide [40], it is possible that a major fraction of cytoplasmic grains correspond to partially hydrolized 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 efficiently cross-link 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-dependant 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 whether the process involves internalization of the ligand alone or, as observed in other systems [1],[2],[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 certain 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°C 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 h of incubation with ^{3}H-NT at 37°C, 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 induces 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, further suggests that either the ligand, receptor-ligand complexes, or metabolites thereof, may be involved, perhaps through a nuclear translocation process, into long-term genomic effects of NT on its target cells [49],[50].

FIGURE LEGENDS

Figure 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°C for one h to ensure saturation of cell surface receptors, after which they were transferred to 37°C 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. (From [33]).

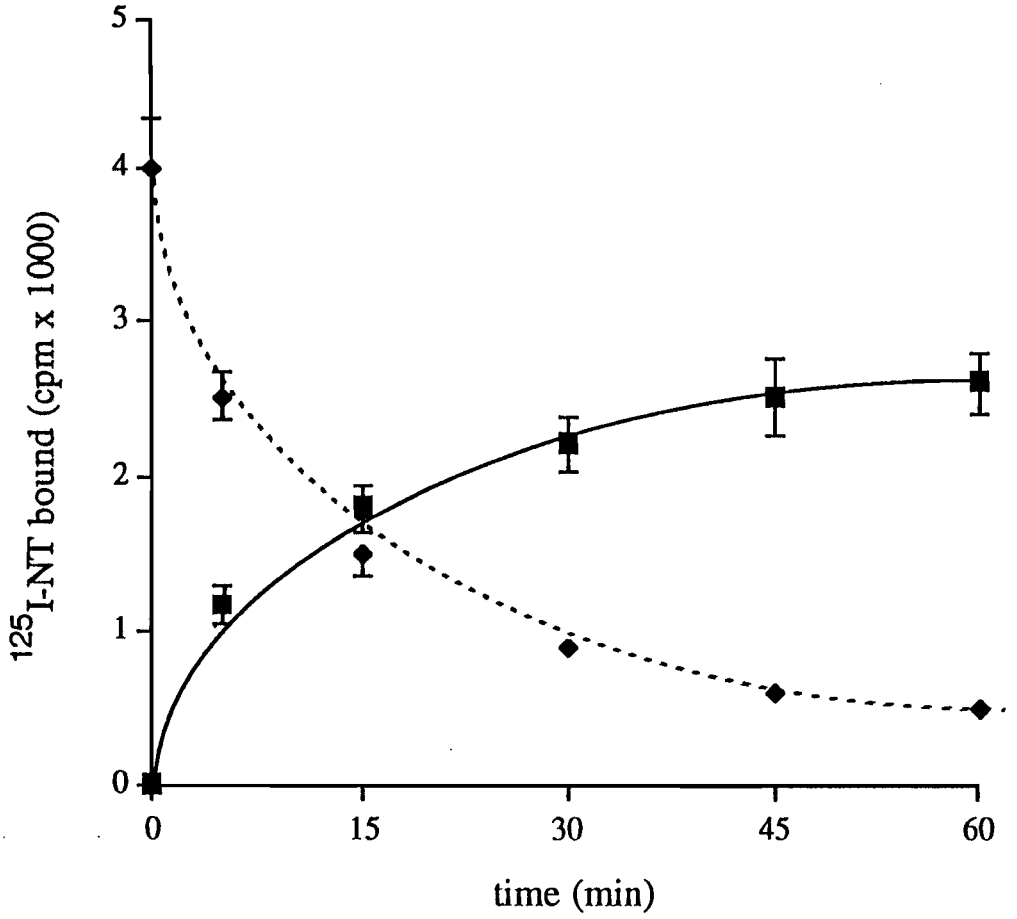
5
4
3
2
1
0
^{125}I-NT bound (cpm x 1000)
0
15
30
45
60
time (min)

Figure 2

Dependence of ^{125}I-NT internalization of the concentration of extracellular NT. Cells were incubated with increasing concentrations of ^{125}I-NT for 45 min at 37°C 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 3 independent experiments carried out in duplicate. (From [34]).

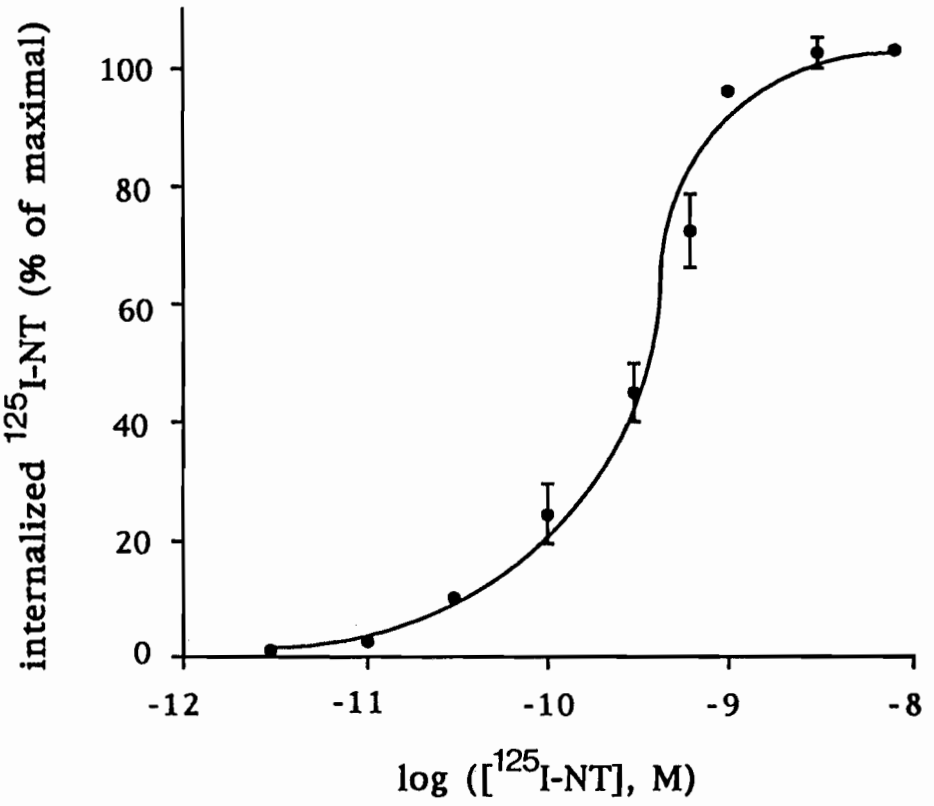
internalized ^{125}I-NT (% of maximal)
100
80
60
40
20
0
-12
-11
-10
-9
-8
log ([^{125}I-NT], M)

Figure 3

Light microscopic autoradiograms of neurons labeled in culture after incubation with 0.2 nM ^{125}I-NT at 37°C. **a.** Cells processed whole after 5 min of exposure to the radioligand. Note the selective accumulation of silver grains over 3 fusiform neuronal perikarya and their processes. **b.** Autoradiogram of a 1μm-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. (From [13]).

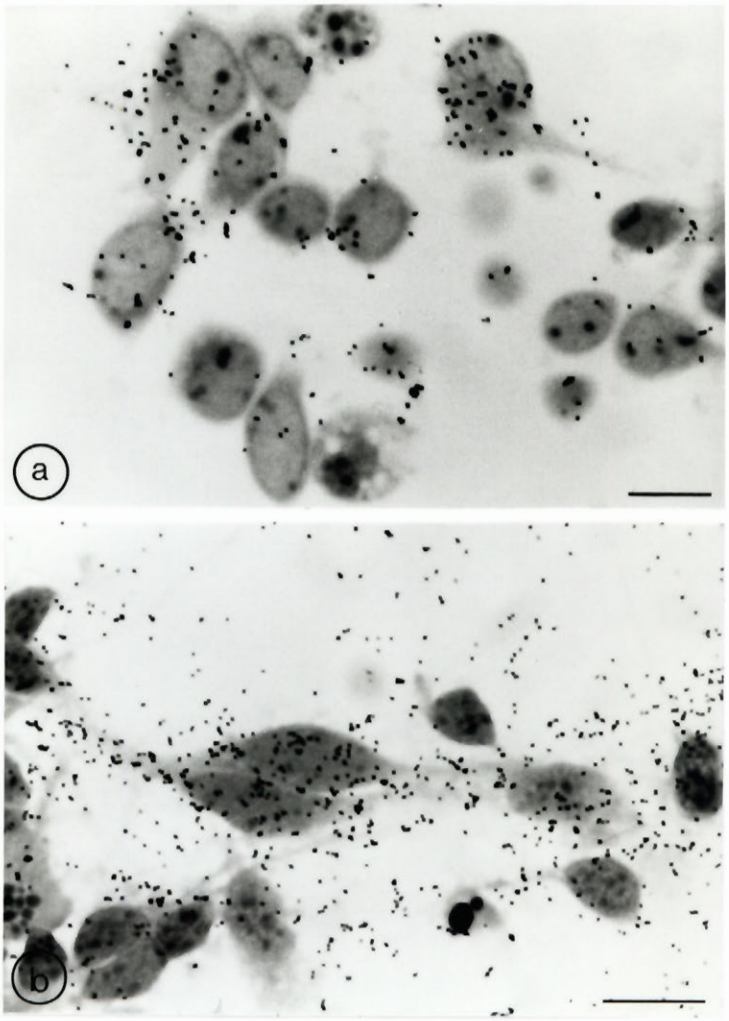
a
b

Figure 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°C or 10°C or after 30 min of incubation at 10°C. Labeling of perikarya expressed as number of grains per unit area. Labeling of processes expressed as number of grains per unit length. (From [13]).

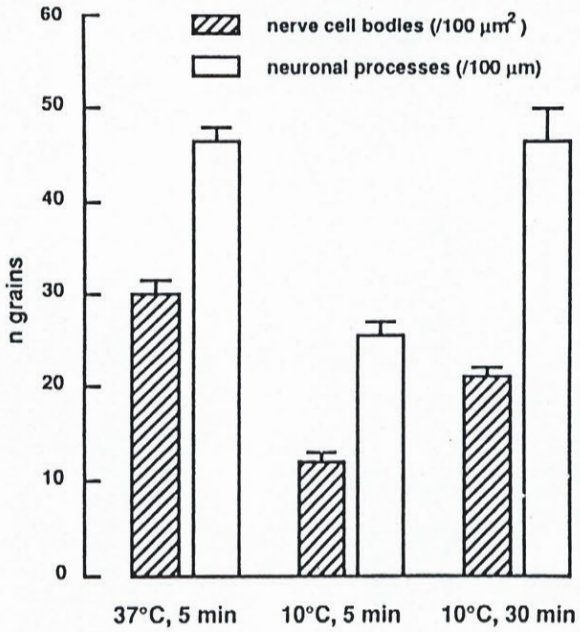
60
50
40
30
20
10
0
n grains
nerve cell bodies (/100 µm²)
neuronal processes (/100 µm)
37°C, 5 min
10°C, 5 min
10°C, 30 min

Figure 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 FITC and Texas red: 488 nm. Scale bars: 10 μm.

a. SN17 hybrid cell incubated for 45 min at 4°C 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°C) 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°C. 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 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 8 h (g,h) 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 h is similar to that observed after 30 min of exposure to the ligand in culture. At 8 h, labeled intracellular

granules are markedly larger and clustered against the nuclear membrane (g,h). Note the absence of nuclear labeling.

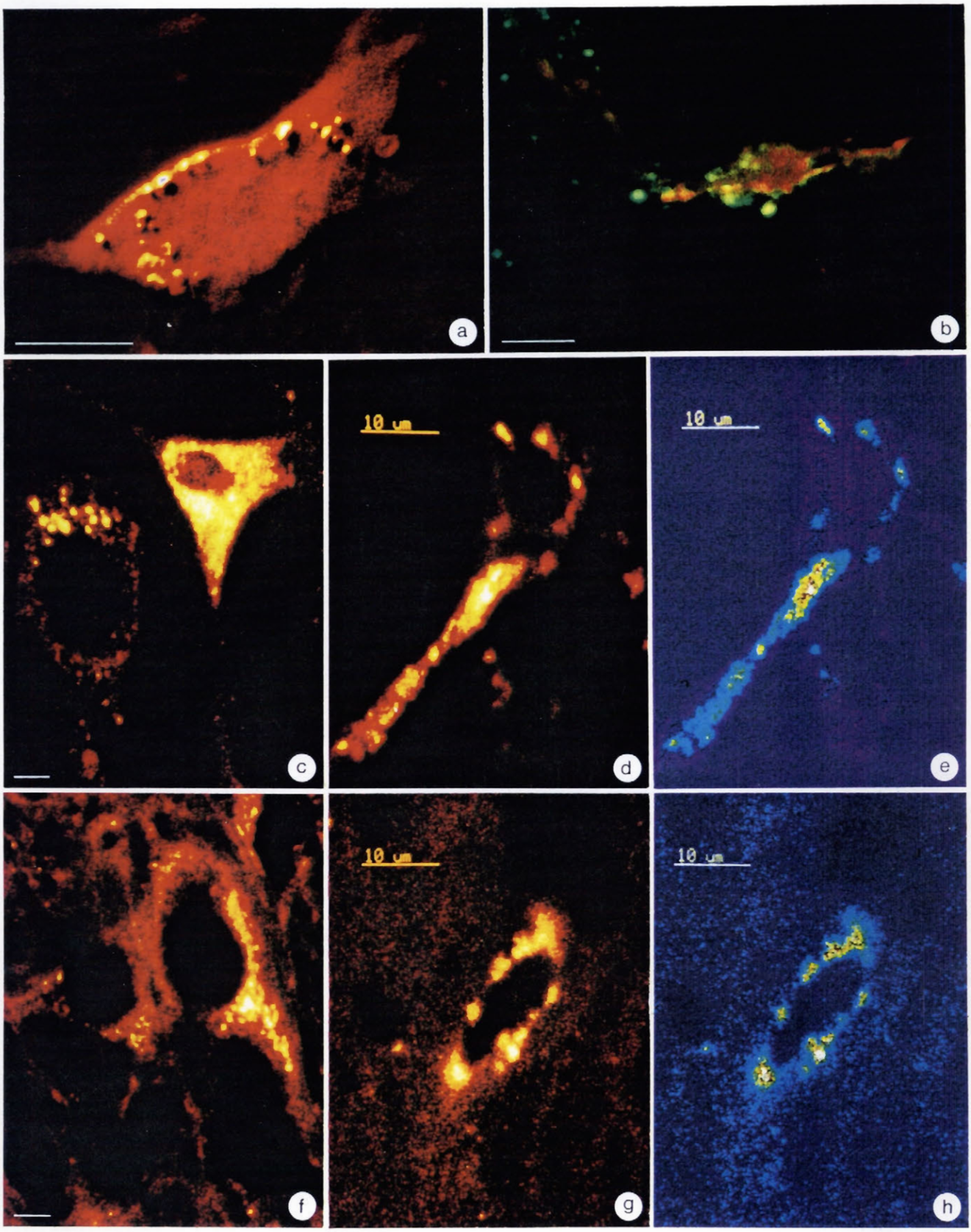
a
b
c
10 um
d
10 um
e
f
10 um
g
10 um
h

Figure 6

Electron microscopic autoradiograms of nigral neurons 4.5 h 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.

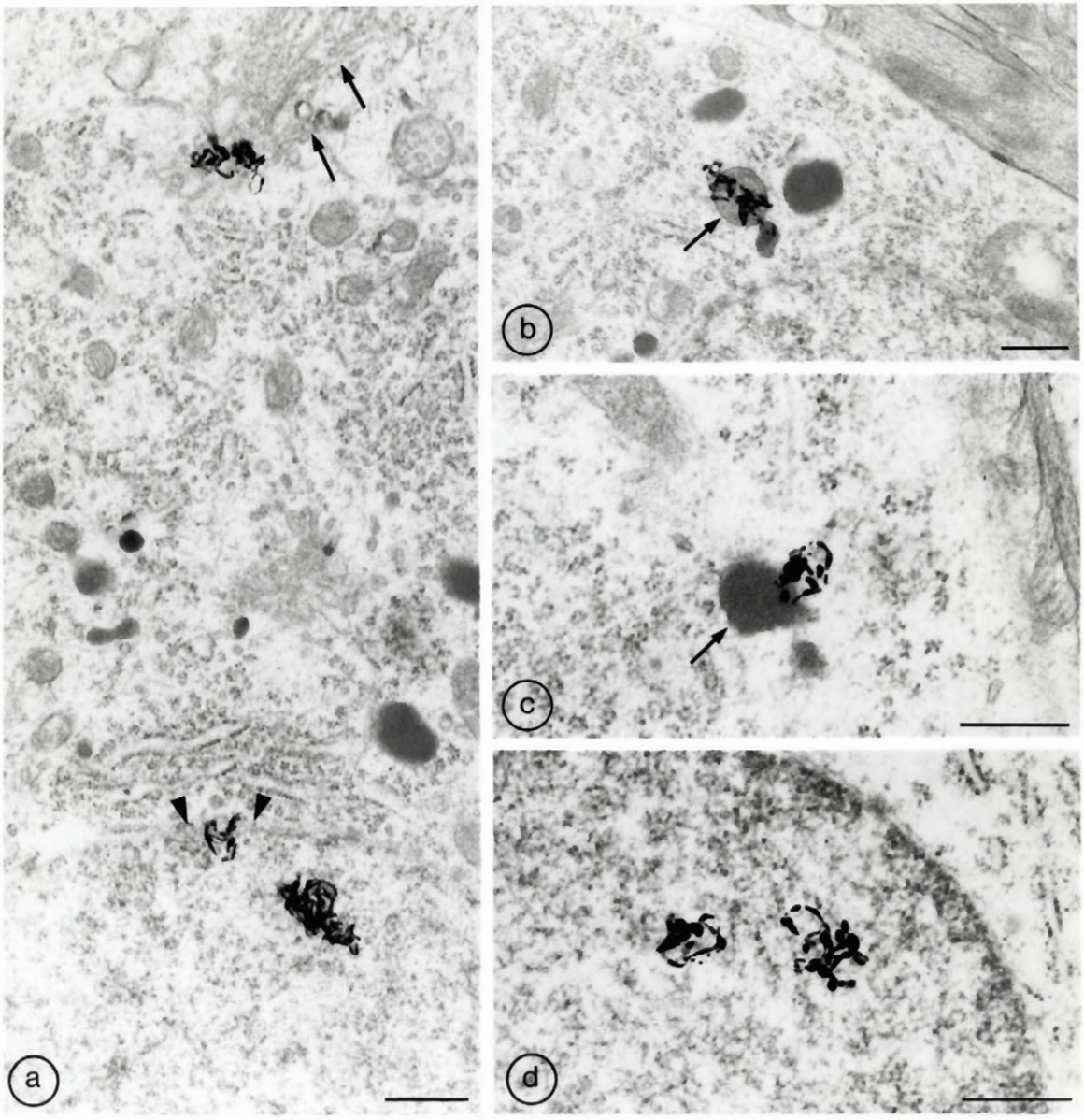
a
b
c
d

Figure 7

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

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

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