Maturation of the Neurotensin/Neuromedin N Precursor in the Central Nervous System of the Rat.

by

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LIST OF ABBREVIATIONS

NT: Neurotensin

NN: Neuromedin

FITC: Fluorescein Isothiocyanate

TR: Texas Red

CLSM: Confocal Laser Scanning Microscope

PAP: Peroxidase anti-peroxidase

RER: Rough endoplasmic reticulum

TGN: Trans Golgi network

POMC: Pro-Opiomelanocortin

K6L, K7Y,E6I, Y7I: The abbreviations for synthetic tyrosine-extended or unmodified precursor peptide fragments, containing the first letter corresponding to the single letter code of the first amino acid, a middle number indicating the number of peptide residues and a last letter corresponding to the single letter code of the last amino acid.

ABSTRACT

The NT/NN precursor molecule possesses four lysine-arginine dibasic residues which represent potential sites of cleavage by proteolytic maturation enzymes. Knowing that all of these dibasic residues are cleaved to a variable extent in rat brain, the aim of the present study was to identify immunohistochemically the regional, cellular and subcellular localization of the various polypeptide intermediates resulting from the precursor's intraneuronal processing and to attempt to determine which enzymes are involved in this processing. We have used site specific antibodies directed against NT, against the exposed KLPLVL(K6L) and EKEEVI(E6I) sequences of the precursor as well as against the PC1 and PC2 maturation enzymes. In a first set of experiments, each of the NT, E6I and K6L antigen was singly labeled in serial sections through the rat brain using a PAP procedure. In a second series of experiments, NT and either E6I or K6L antigens were labeled in pairs using immunofluorescence and visualized at low and high resolution by confocal microscopy. In a third series of experiments, antibodies against NT and against the protein convertases PC1 and PC2 were used in serial sections through the rat forebain to determine the regional correspondence between the three antigens. In the first series of experiments, E6I and K6L immunoreactivity was essentially confined to nerve cell bodies and terminals in area displaying NT immunoreactivity. In the absence of colchicine pre-treatment, the NT and E6I immunostained perikarya remained sparse throughout, whereas those with K6L were virtually undetectable By contrast, terminal immunostaining was intense for both NT and E6I in most regions examined, with K6L remaining consistently weaker except in the globus pallidus where both the E6I and the K6L immunoreactive arbors were more widespread than those of NT. The

colchicine pretreatment markedly increased the number of NT and E6I cell bodies but only marginally augmented the number of K6L immunoreactive perikarya (which remained sparse throughout). Interpreted in terms of both the pattern and the temporal sequence of the maturation process, the results suggest that the cleavage of the dibasic sites adjacent to the E6I and K6L sequences is more extensive in certain brain regions than in others and occurs further distal to the cell body than the one giving rise to NT. In double labeling experiments, E6I and K6L antigens were found to be present in the same cells as NT, indicating that even if it is quantitatively different among brain regions, the basic pattern of NT/NN precursor processing remains qualitatively similar throughout the brain. At the subcellular level, a granular pattern of NT and E6I immunofluorescence was distributed throughout the cytoplasm of the perikarya and proximal dendrites. The combination of individual NT and E6I images confirmed the colocalization of these two antigens within the same vesicle-like compartments, suggesting cleavage of the precursor following budding of the secretory vesicle from the trans Golgi network. Comparative analysis of the distribution of PC1 and PC2 with that of NT revealed only a partial overlap of the three markers in the rat CNS. Immunoreactivity for both PC1 and PC2 was mainly concentrated in nerve cell bodies and dendritic processes. In some regions, but not in others, the cytological features and cell distribution of PC2, but not of PC1, containing neurons was comparable to that of NT immunoreactive neurons. In other areas, NT immunoreactive neurons were detected in the absence of either PC1- or PC2-immunoreactivity indicating that other proteolytic enzymes may be involved in the processing of the NT/NN precursor. Taken together, our observations suggest a possible involvement of PC2 but not of PC1 in the proteolytic

processing of the NT/NN precursor.

RÉSUMÉ

Le précurseur commun à la NT et à la NN possède quatre doublets basiques lysine-arginine susceptibles d'être coupés par des enzymes de maturation protéolytique. Sachant que chacun de ces doublets est clivé de façon non homogène dans le cerveau du rat, le but du présent travail a été d'identifier par immunocytochimie la localisation régionale, cellulaire et sub-cellulaire des différents peptides dérivés de la maturation intraneuronale du précurseur, et de déterminer quel(s) enzyme(s) pourrait(ent) être impliqué(s) dans cette maturation. Nous avons utilisé des anticorps spécifiquement dirigés contre la NT, les séquences (libres) KLPLVL (K6L) et EKEEVI (E6I) du précurseur et les enzymes de maturation PC1 et PC2. Dans une première série d'expériences, chacun des antigènes NT, E6I et K6L a été localisé dans des sections adjacentes du cerveau du rat par la technique peroxidase-anti-peroxidase. Dans une deuxième série d'expériences, les antigènes NT et E6I ou K6L ont été étudiés par paire par immunofluorescence et visualisés à faible et à forte résolution par microscopie confocale. Dans une troisième série d'expériences, les anticorps dirigés contre la NT et les enzymes PC1 et PC2 ont été utilisés sur des sections sériées du cerveau antérieur du rat afin de déterminer la correspondance topographique entre ces trois antigènes. Nos résultats ont montré que l'immunoréactivité E6I et K6L était essentiellement confinée aux corps cellulaires et aux terminaisons marqués pour la NT. Sans traitement par la colchicine, peu de corps cellulaires étaient immunologiquement marquées pour la NT et l'E6I alors que les corps cellulaires K6L étaient virtuellement non detectables. Par contre, un marquage immunocytochimique des terminaisons nerveuses a été détecté avec

les anticorps dirigés contre la NT et contre la séquence E6I alors que le marquage K6L était considérablement plus faible, sauf dans la région du globus pallidus où l'arborisation détectée avec l'un ou l'autre des anticorps anti-E6I et anti-K6L était plus étendue que celle de la NT. Le prétraitement par la colchicine a considérablement augmenté le nombre de corps cellulaires immunoréactifs pour la NT et l'E6I mais n'a augmenté que marginallement le nombre de corps cellulaires marqués avec l'antisérum dirigé contre K6L. Interpretés en termes de distribution des différents dérivés de maturation, ces résultats suggèrent que le clivage des doublets dibasiques adjacents aux séquences E6I et K6L du précurseur est dans un premier temps plus marqué dans certaines régions cérébrales que dans d'autres. Quant à la séquence temporelle du processus de maturation, elle semble intervenir au cours du transport axonal du précurseur NT/NN vers les terminaisons nerveuses. Les études de double marquage ont démontrées que les antigènes E6I et K6L sont présents dans les mêmes cellules que la NT, indiquant que même si la maturation de base du précurseur commun à la NT et à la NN est quantitativement différent d'une région à une autre, il demeure qualitativement le même. Au niveau sub-cellulaire, un marquage granulaire correspondant à la NT et au E6I était distribué dans le cytoplasme des corps cellulaires et des dendrites proximaux. La combinaison des images acquises individuellement pour la NT et le E6I a confirmé la colocalisation de ces deux antigènes au niveau d'organelles ressemblant à des vésicules de sécrétion. Ceci suggère que la maturation du précurseur commun à la NT et à la NN se produit suite à la libération des vésicules de sécrétion au niveau du complexe *trans* golgien. L'analyse comparative de la distribution de PC1 et PC2 avec celle de la NT a révélé une superposition partielle des trois antigènes dans la cerveau antérieur du rat.

L'immunoréactivité pour PC1 et PC2 était principalement concentrée dans les corps cellulaires et les fibres dendritiques. Dans certaines régions, la distribution des neurones contenant PC2, mais non PC1, était comparable à celle des neurones contenant la NT. Dans d'autres régions, la prépondérance de neurones contenant la NT malgré l'absence d'immunoréactivité pour l'un ou l'autre des enzymes PC1 et PC2 indique que d'autres enzymes protéolitiques sont impliqués dans la maturation du précurseur. Conjointement, nos observations suggèrent une implication possible de PC2, et non de PC1, dans la maturation protéolitique du précurseur commun à la NT et à la NN.

INTRODUCTION

The existence, in the central nervous system of a variety of peptides that play the role of neurotransmitter and/or neuromodulator is well established. It has been recognized for some time that the synthesis of these peptides results from the processing of a relatively limited number of precursor molecules often common to several peptides. The transcriptional variability and post-translational maturation of these precursors lead to a diversity of molecules in the nervous system which provides the flexibility essential to biological adaptation and survival of the living organism.

So far, the biological activity of unprocessed neuropeptide precursors and of many of their post-translational maturation products has remained elusive. This is largely due to the lack of appropriate techniques for their characterization and identification. In fact, many neuropeptides have been discovered almost by chance thanks to unexpected functional properties of partially purified extracts (Carraway and Leeman 1973). Insight in the mechanism of neuropeptide maturation has grown explosively with the recent advances in molecular cloning, recombinant DNA and mutagenesis. These approaches have greatly facilitated the task of identifying and determining the primary structure of pro-peptides, and have made possible the generation of specific probes and antibodies for the study of their maturation.

Principles of pro-peptide maturation

The expression by peptidergic neurons of the genetic information coding for biologically active neuropeptides involves a cascade of highly organized processes. The biosynthetic process begins with the transcription of the gene into a molecule of mRNA, followed by its translation on the rough endoplasmic reticulum (RER) into a precursor protein. The precursor is then transported into RER cisternae, translocated to the Golgi apparatus and ultimately packaged into the final membrane-bound compartment, the secretory vesicle (Gainer, 1985; Loh, 1984).

Studies on the biosynthesis of neuropeptide precursors in the central and peripheral nervous systems have shown that the propeptide itself is generally biologically inactive and must undergo a series of post-translational modifications to yield biologically active molecules (Jung, 1991; Loh, 1983). These modifications take place during the transport of the precursor through the membrane system of the cell. In the RER, the precursor undergoes removal of the signal peptide, glycosylation and formation of disulfide bonds. In the Golgi, more complex glycosylation takes place together with phosphorylation of the precursor (Castel, 1984). The above post-translational events are used to complete the precursor's structure but do not involve the endoproteolytic and enzymatic steps which produce the final peptides. The enzymatic events which generate the active molecules include both limited proteolysis and functional modifications of the generated peptide such as N-terminal acetylation and pyrolation as well as C-terminal carboxylation and amidation (Harris, 1989). These events are induced by the cleavage

of the chain at appropriate peptide bonds (Docherty, 1982). Although the organization of cleavage sites appears to be somewhat diverse, careful analysis of precursor sequences collected in data banks reveals that, in organisms as diverse as yeasts and mammals, doublets of basic amino acids bracketing the peptide hormone are the most common sites of endoproteolytic cleavage to generate the final secretory products (Cohen, 1987; Rholam, 1986). In fact, most pro-hormones/pro-neuropeptides have pairs of basic amino acid residues flanking the bioactive sequences, the most common being Arg-Arg and Lys-Arg, although Arg-Lys and Lys-Lys can also be found (Loh, 1984; Mains, 1987; Mathis, 1992). There are two exceptions to this rule. One, unprocessed doublets of basic residues often occur in precursor proteins. Two, processing at mono-, tri- and tetrabasic sites have also been found to yield a minority of bioactive peptides (Smeekens, 1993).

Although the primary sequence of the pro-protein is critical for the processing pattern, the conformation of the molecule also plays a role. Results from different groups indicate that in many pro-hormones the entire recognition site encompasses a sequence of several residues, including the dibasic bonds. More specifically, cleavage may require that the basic residues be positioned in or immediately adjacent to a ß-turn (Rholam, 1986). The primary amino acid sequence of precursor proteins would then be responsible for a particular tridimentional conformation of the molecule that exposes selected cleavage sites to the action of the processing enzymes, thus determining cleavage specificity (Rangaraju, 1991). Consequently, the deduced pre-propeptide sequence does not necessarily predict the sites of proteolytic processing.

After endoproteolytic cleavage at the carboxyl-terminal side of the dibasic doublet, the next steps in peptide maturation involve the removal of the basic residues from the carboxyl terminal of the cleavage site and the amidation of this same terminus (Fig.1) (Eipper, 1983).

Various lines of evidence point to secretory vesicles as primary sites for endoproteolytic pro-protein cleavage (Gainer, 1985; Loh, 1983). Thus, it is currently believed that the initial endoproteolytic events occur upon formation of secretory vesicles and that further maturation takes place during axonal transport (Browstein, 1989). For instance, studies of the precursor of oxytocin and vasopressin have shown that from their perikaryal sites of synthesis in the supraoptic nucleus and paraventricular nuclei, the precursors are transported in secretory vesicles to the cell's axon terminal in the median eminence wherein the conversion of the precursors to the biologically active peptide products takes place (Gainer, 1985; Harris, 1989). In keeping with these observations, enzymological studies have located and characterized putative processing enzymes in secretory vesicles (Rouille, 1992).

However, recent evidence from pulse-chase experiments in combination with subcellular fractionation and/or autoradiography suggest that the processing of precursor proteins may be initiated in the *trans* region of the Golgi complex, where the sorting and packaging of the secretory components have been shown to take place (Fisher, 1988; Habener 1981; Lepage-Lezin, 1991; Rangaraju, 1991). Thus, endoproteolytic cleavages occurring in the *trans*-Golgi network have been demonstrated for the precursor of the egg-laying hormone, in the bag cell cluster of *Aplysia californica* (Fisher, 1988). Each

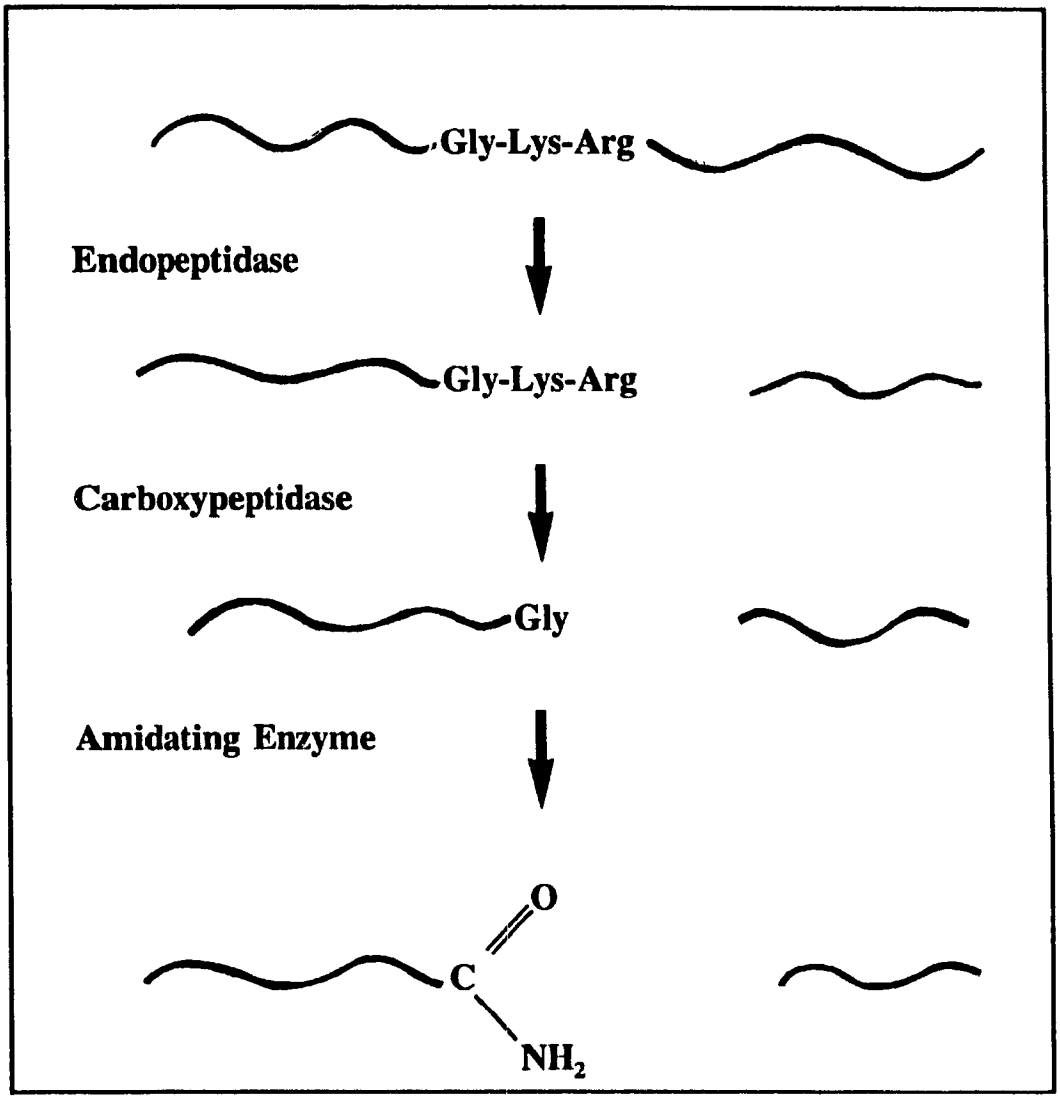


Figure 1. Steps in propeptide maturation. (Modified from Sossin, 1989)

neuron in the bag cell clusters synthesizes several peptides derived from a single pro-hormone and packages them at the *trans* Golgi level into separate vesicles. These vesicles are then differentially targeted to specific neuronal processes, thereby spatially segregating peptides with different biological activities to different regions of the neuron (Sossin, 1990). Therefore, an important issue that arises when a precursor is known to give rise to more than one biologically active peptide is where in the cell these peptides are generated and whether they are stored in the same vesicles.

The processing enzymes

Only a few proteolytic enzymes have so far been characterized and there is still little knowledge of their physiological functions *[in vivo]*. Since many of these enzymes occur in quantities too small for isolation, the methods of gene cloning and mutagenesis have provided precious tools for the characterization of these enzymes and the elucidation of their mode of action. Among the many functions of proteolytic enzymes, the selective cleavage of peptide precursors into active fragments is of primary interest. As discussed in the previous section, two types of enzymatic activity are necessary for the conversion of the pro-protein in the final products (Fig.1). The first implies a protein convertase which cleaves primarily at pairs of basic amino acids. The second rests on the action of a carboxypeptidase and an amidating enzyme which provide for the removal of basic residues and the amidation of the C-terminus, respectively. Thus, after endoproteolytic cleavage, a carboxypeptidase E trims the basic residue extension at the C-terminus of

the peptide (Fricker, 1986). Peptides that have a glycine residue preceeding the basic cleavage site can be further modified by removal of the glycine by a specialized enzyme (eg. peptidyl-glycine-α-amidating monooxygenase, PAM) generating an amidated carboxyl group (Eipper, 1987; Sossin, 1989). Both modifications are essential for the bioactivity of many neuropeptides, the later being particularly important in protecting the peptide from degradation by enzymes that act on the COOH-terminus (Jung, 1991). Recently, a family of protein convertase (PC) enzymes has been purified and characterized. These convertases have been shown to be involved in the initial endoproteolytic cleavage of a number of pro-peptide molecules in both the brain and peripheral tissues.

Identification of the prohormone convertases was first made possible by the cloning of the yeast enzyme Kexin (Kex-2) and the demonstration that this enzyme processes mammalian pro-proteins both *in vitro* and *in vivo* (Julius, 1984). Kex-2 is a calcium-dependent serine protease with a significant degree of homology with the subtilisins, a class of bacterial serine proteases (Fuller, 1989). Using Kex-2 as a prototype for mammalian convertase, it was demonstrated that a human furin gene product might represent the mammalian counterpart of Kex-2 (Van den Ouweland, 1990). The combination of polymerase chain reaction and degenerate probes directed against the catalytically important sequences within Kex-2 and furin were to allow identification of five additional mammalian homologues of furin: prohormone convertase 1 (PC1) (Seidah, 1991) also known as PC3 (Smeekens, 1991), PC2 (Seidah, 1990), PACE4 (Kiefer, 1991), PC4 (Nakayama, 1992) and PC5 (Lusson, 1993).

These members of the subtilisin/kexin family were found, by *in situ* hybridization and Northern blot analysis, to have a tissue specific distribution. Furin and PACE4 exhibit a ubiquitous distribution with high concentrations in liver and kidney whereas PC4 is primarily expressed within ovarian and testicular germ cells (Nakayama, 1992).

PC1 and PC2 are found only in cells of endocrine and neuroendocrine origin (Seidah, 1991; Smeekens, 1992). In the brain, immunocytochemical studies have confirmed that PC1 and PC2 are exclusively localized in neurons, where they are present both in perikarya and processes (Seidah, 1991). Preparations from endocrine tissue, i.e. adrenal chromaffin cells, have shown that PC1 and PC2 are present within secretory granules. Moreover, the activity of both enzymes is optimal at the acidic pH (5.5-6.5) found within the secretory granule (Kirchmair, 1992b; Smeekens 1992).

In addition to exhibiting exquisite selectivity for pairs of basic residues of the type Lys-Arg and Arg-Arg (Benjannet, 1991; Seidah, 1992) PC1 and PC2 have been shown to be substrate specific (Rangaraju, 1991). For instance, when co-expressed with pro-opiomelanocortin (POMC) in AtT-20 cells, PC2 preferentially processed POMC to ß-endorphin and α-MSH, whereas PC1 processed it to ACTH, ß-lipoprotein (ß-LPH) and some ß-endorphin; i.e. the same two sets peptides known to be produced *in vivo* by intermediate and anterior pituitary cells, respectively (Benjannet, 1991; Marcinkiewicz 1993). One implication of the distinctive cleavage specificity of PC1 and PC2 is that excision of maturation products from the parent precursor depends on the tissue specific expression of specialized processing enzymes. Thus, a difference in the relative expression levels of PC1 and PC2 could explain the occurence of tissue-specific

differences in the processing of their substrate (Eipper, 1987; Seidah, 1992).

The emerging characteristics of the PC1 and PC2 endoproteases, including their tissue-specific expression, cleavage site selectivity and substrate specificity indicate that they could represent endoproteases specifically functioning within the characteristic regulated pathway of neuroendocrine cells (Smeekens, 1993). Additional support for their role as endoproteolytic enzymes in neuropeptide processing comes from their selective expression within peptide-rich brain regions (Cullinan, 1991). For example, the peptide rich hilar neurons of the dentate gyrus have been shown by *in situ* hybridization to express a high level of PC2 and a moderate level of PC1. High levels of PC1 and PC2 mRNA were also seen within peptide-rich forebrain structures such as the amygdaloid body and the bed nucleus of the stria terminalis. In the hypothalamus, the highest levels of expression of PC1 and PC2 transcripts were detected in the paraventricular and suprachiasmatic nucleus, in which neuropeptides such as oxytocin, vasopressin and CRH are synthesized (Cullinan, 1991).

It remains unknown however, whether there is a strict association of PC1 and PC2 with specific neuropeptidergic systems or whether these two enzymes act in a more widespread fashion as is the case for furin (Day, 1993). Nevertheless, the detection of different combinations of the convertases in various brain regions suggests that "specific enzymatic pathways" are involved in the processing of neuropeptide precursors (Cullinan, 1991). An approach to clarify this issue would be to carry out experiments in well defined peptidergic systems to detect the potential colocalization of PC1 and PC2 with the final maturation products of these systems.

The NT/NN Precursor and its Derivatives

Neurotensin

Initially discovered in bovine hypothalamic extracts by Carraway and Leeman (1973), neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) is a ubiquitous tridecapeptide present in almost all vertebrates and even in primitive species such as protozoans (Bhatnagar, 1981; Carraway, 1973). Neurotensin was purified at the same time as substance P following the observation that intravenous administration of partially purified hypothalamic extracts enhanced vascular permeability and induced a characteristic vasodilation, particularly in the ears and the face area (Carraway, 1973). Subsequent to its isolation from the brain and peripheral tissues in a variety of species, the primary amino acid sequence were deduced and found to be evolutionary conserved at the C-terminal region (Carraway, 1982). Evidence rapidly accumulated in support of a neurotransmitter role for NT in the central nervous system. The most important criteria are listed below:

i. Expression in selective neuronal populations

ii. Ca^{++}-dependent release by depolarizing agents

iii. Production of physiological effects

iv. Rapid degradation by peptidases

v. Specific binding to high affinity sites

i. *Expression in selective neuronal populations*

Radioimmunoassay (Carraway, 1973) and immunocytochemical techniques (Jennes, 1982; Khan, 1980) were used to examine the topographic and cellular distribution of NT. A widespread and heterogeneous distribution of NT-immunoreactive signal was detected by radioimmunoassays throughout the central nervous system of several mammalian species. High concentrations of NT were thus detected in the amygdala (central and medial nucleus), nucleus accumbens, lateral septum and several regions of the hypothalamus, including the arcuate, periventricular and the suprachiasmatic nucleus. Moderate concentrations of NT were found in the basal ganglia (striatum), substantia nigra, the ventral tegmental area and the periaqueductal grey matter (Jennes, 1982; Uhl 1982). By immunocytochemistry, NT was shown to be concentrated in selective populations of neuronal perikarya, dendrites, axons and axon terminals (Jennes, 1982). NT-containing systems so far identified in the rat include pathways: 1) from the ventral tegmental area to the nucleus accumbens (Kalivas, 1984); 2) from the central nucleus of the amygdala to the bed nucleus of the stria terminalis (Uhl 1979); 3) from the arcuate nucleus of the hypothalamus to the median eminence (Ibata, 1984; Jennes, 1982); 4) from the subiculum of the hippocampus to the alveus, fimbria and mammilary bodies (Sakamoto, 1986).

ii. *Ca^{++}-dependent release by depolarizing agents*

It has been demonstrated from rat hypothalamic slices *in vitro* (Iversen, 1978) that NT can be released by depolarizing concentrations of external potassium in a calcium-dependent manner. Furthermore, NT has been shown to be released from anterior pituitary cells following stimulation (Carraway, 1976).

iii. ***Production of physiological effects***

When directly injected into cerebral tissue or into the lateral ventricle, NT was shown to affect thermoregulation, nociception, locomotor behaviors, muscle tone, sleep as well as feeding and satiety (Bissette, 1976; Castel, 1989; DeBeaurepaire, 1988; Jolicoeur, 1981; Nemeroff, 1979; Oshbar, 1979). It is noteworthy that NT shares many pharmacobehavioral and electrophysological characteristics with antipsychotic drugs. Thus, both NT and antipsychotics potentiate barbiturate- and ethanol-induced sedation and both produce hypothermia, muscle relaxation and decrease locomotor activity (Bissette, 1976; Nemeroff, 1980). These antipsychotic properties of NT were the first indications to suggests a possible interaction between NT and dopamine. The subsequent demonstration that NT modulates dopaminergic activity (for a review, see (Kasckow, 1991)) and mimics some of the physiological and behavioral effects induced by DA antagonists (for review (Studler, 1988)), lead Nemeroff to consider NT as a potential endogenous neuroleptic (Nemeroff, 1980). However, recent data suggests that the antipsychotic properties of NT and its effects on the dopaminergic system are not so simple and most probably result from an interaction at the molecular and/or receptor

and/or second messenger levels (Adachi,1990; Agnati, 1983; Amar, 1987; Nouel, 1992).

iv. *Rapid degradation by peptidases*

Neurotensin is rapidly degraded to inactive fragments when exposed to both soluble and particulate fractions of brain tissue preparations (Checler, 1986; Checler 1983a; Checler, 1985). The enzymatic inactivation results from three primary cleavages each of them mediated by one of several metallo-endopeptidases: metallo-endopeptidase 24.11 (enkephalinase), 24.15 and 24.16. A distinct set of enzymes is responsible for the secondary cleavage of the generated fragments. The concentration and activity of the metallo-peptidases was shown to differ according to the cerebral region under study (Davis, 1992). These observations confirmed that a differential rate of NT degradation exists in different cerebral regions (Checler, 1991; Davis, 1992).

v. *Specific binding to high affinity sites.*

The use of high specific activity radio-iodinated ligand rendered possible the delineation of two independent classes of saturable and reversible specific NT binding sites in mammalian brain (Mazella, 1988; Mazella, 1985; Sadoul, 1984): a high affinity site (Kd = 1.6 nM) which displays relatively low binding capacity (Bmax = 16.3 fmol/mg) (Kitabgi, 1985) and a low affinity site (Kd = 4-7 nM) with a high capacity (Bmax = 37,2 fmol/mg). Another discriminating characteristic of the low affinity receptor is that it is

sensitive to the H1 antihistamine levocabastine (Kitabgi and others 1987; Schotte, 1986; Schotte and others 1988). On the basis of its low saturability and relatively ubiquitous distribution in all murine brain regions (Kitabgi, 1987; Schotte, 1986) this low-affinity binding site is thought to represent an acceptor site for NT. In contrast, the high-affinity, saturability and heterogenous distribution of the high-affinity site are characteristics of a functional receptor. In fact, it was shown that the high affinity NT receptor was the only binding site that was coupled to a modulation of intracellular events, i.e cAMP, cGMP and phosphatidyl inositol turnover (for review see (Vincent, 1990).

NT/NN precursor

Many studies have been published on the maturation of polypeptidic precursors in the mammalian CNS (Khachaturian, 1985; Liston, 1984; White, 1986) but only a few have been carried out on the Neurotensin/Neuromedin N (NT/NN) precursor. The cDNA encoding the neurotensin molecule was first cloned from a canine enteric mucosa cell library and later from bovine hypothalamus cDNA (Dobner, 1987; Kislauskis, 1988). The protein precursor sequences predicted from bovine hypothalamus and canine intestine cDNA clones vary at only 9 of the 170 amino acids residues suggesting that within a given species, identical precursors are synthesized in both the intestine and the central nervous system (Kislauskis, 1988).

The rat NT/NN gene coding region is divided into 4 exons and 3 introns. The NT and NN coding domains are located in tandem on exon 4 near the carboxyl terminus of the predicted precursor (Kislauskis, 1988). As can be seen in Fig. 2, the NT/NN

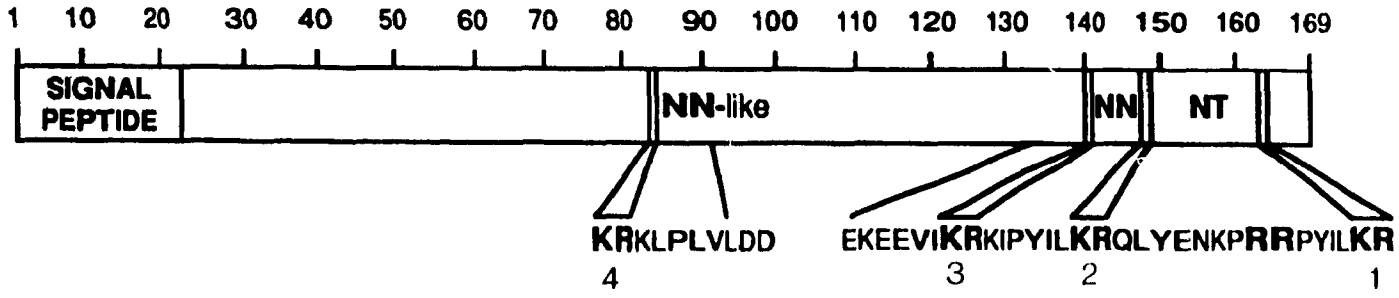


Figure 2. Schematic representation of the NT/NN precursor. Peptide sequences of interest are written according to the one letter amino acid code, and are derived from the rat precursor sequence.

precursor possesses four pairs of Lys-Arg dibasic residues which represent putative sites of cleavage for proteolytic maturation enzymes. Processing of the three doublets that are the closest to the C-terminal extremity of the precursor releases one copy each of NT (Carraway, 1973) and NN (Minamino, 1984). The sequence adjacent to the first dibasic bond is a NN-like sequence and is therefore potentially endowed with biological activity. The presence of this fourth doublet implies that the post-translational processing which gives rise to NT and NN may concomitantly generate several alternative intermediates and final products. In order to understand the regulatory function of these two neuropeptides and possibly of other peptides not yet characterized, it is mandatory to understand the biosynthetic mechanisms acting upon their precursor.

Neuromedin N

The C-terminal extremity of NT exhibits substantial sequence homology with the hexapeptide NN, derived from the same precursor (Minamino, 1984). Isolated from porcine spinal cord and characterized on the basis of its gut contracting effects, NN possesses a high affinity (although 4x lower than that of NT) for the central receptors of NT (Checler, 1986a). When protected from degradation by peptidase inhibitors, NN produces behavioral and physiological effects similar to those of NT following ventricular and intracerebral administration (Coquerel, 1988; Dubuc, 1988).

A number of findings support the idea of a tissue specific variation in the processing of the NT/NN precursor. For example, different types and/or proportions of maturation products being generated in the brain as compared to the heart, adrenal,

intestine or medullary thyroid carcinoma 6-23 cell line (Carraway, 1990; Carraway, 1993; DeNadai, 1993). Furthermore, comparison of the distribution of immunoreactive NN and NT in micropunched brain structures revealed marked regional variations in the ratio of NT over NN. High ratio values (>3) were found in the globus pallidus, the hypothalamus, the VTA and the substantia nigra pars compacta. Nevertheless, it is reasonable to speculate that in neurons expressing this precursor, the maturation process would give rise to colocalization of these different products. In fact, despite a variation in the NT/NN ratio from one region of the brain to another, NT and NN were found to have a similar regional distribution (Carraway, 1990; DeNadai,1989). Nevertheless, the possibility remains that the processing of the precursor is subject to regional variations as is the case in neurons expressing POMC in the anterior and intermediate lobes of the pituitary (Douglass, 1984).

The distribution of neurons expressing the NT/NN precursor mRNA, as visualized by *in situ* hybridization, largely conforms to that of NT immunoreactive neurons detected by immunohistochemistry following colchicine treatment These observations indicate that in most brain regions, neurons that express the NT/NN precursor also process it to NT. A notable exception is the hippocampus where high levels of NT/NN mRNA hybridization signal are apparent in the CA1 field and in the subiculum (Alexander,1989; Hara, 1982), but no NT immunoreactivity have yet been detected. In addition, although the NT content of the hypothalamus is 30 times that of the cortex, the NT/NN mRNA level in this region is only 5 fold higher than in the cortex (Kislauskis, 1988). It was proposed that hippocampal and cortical neurons expressing high levels of

NT/NN precursor but undetectable or low levels of NT might process the precursor so as to produce NN and/or another maturation product (Alexander, 1989; Kislauskis, 1988). This hypothesis remains to be verified since no immunohistochemical data are available on the distribution of these products in the CNS. Nonetheless, biochemical data suggest that the NT-expressing neurons also give rise to NN, given the extensive co-localization of these two peptides in the brain (Jennes, 1982; Khachaturian, 1985; Liston, 1984).

Principles of Confocal Microscopy

The principle of confocal microscopy rests on the use of laser light from a point source, combining focal illumination of a single point in the specimen with imaging of the illuminated specimen on a detector pinhole (Fine, 1988). Hence, the term confocal is derived from the fact that the objective and the collector lenses are simultaneously focused on the same point in the object of interest (Brakenhoff, 1989). The basic light path and arrangement of the instrument for fluorescence operation are represented in Fig.3 (Carlsson, 1989).

Since only one point is imaged, the laser beam must be scanned across the specimen to image the entire slice onto the detector. Furthermore, the confocal images will appear to be slices of the object because only a thin region of the specimen is viewed at any time (fig.4; Richardson, 1990). A series of optical sections, with a slight shift of focus level between each, may be integrated into a single image, using a simple computer

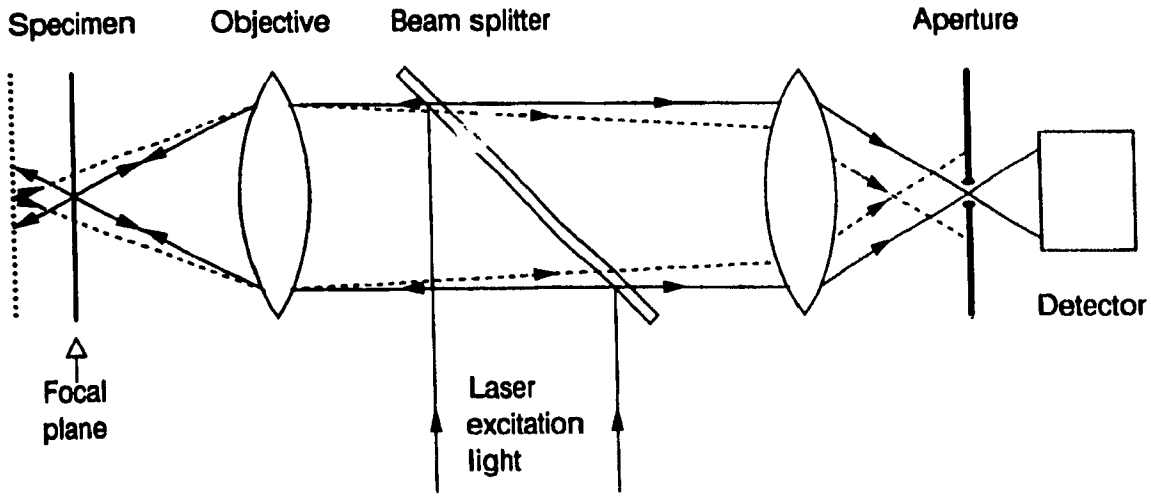


Figure 3. Light originating from the laser illumination pinhole is focused on a certain point in the object. Emitted or reflected light from the illuminated point retraces the incident path (solid lines) through the objective lenses, passing the beam splitter (dichroic mirror in fluorescence) and is then imaged on a second pinhole, the detector aperture. Light coming from structures above or below the plane of focus (dash lines) will not reach the detector, allowing thin optical sections to be made through the specimen. It excludes the majority of residual out-of-focus emission. The detector is connected to a computer which digitize and store the image data for further processing and analysis.

(from Carlsson, 1989)

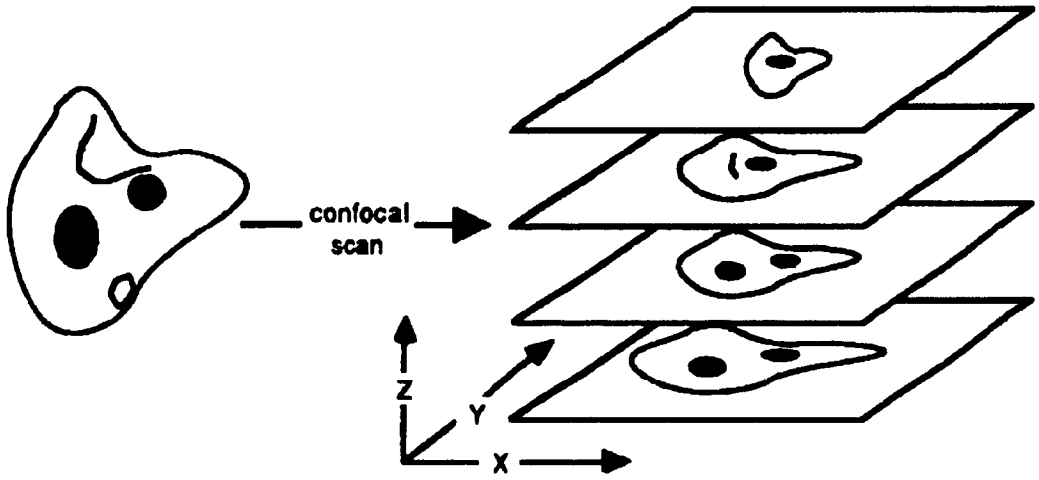


Figure 4. **Confocal scan of a cell. The specimen appears as slices since only a thin segment (in the z plane) is view at any time. The combination of all the slices, i.e. the optical sections, results in an image which contains the full 3-D representation of the specimen.**

(from Richardson, 1990)

algorithm. Essentially, the depth of field of the resulting image is equal the sum of the tickness of the optical sections (Pauley, 1990), and contains a full 3-D representation of the specimen.

Confocal microscopy provides a bridge between conventional light and electron microscopy. It is preferable to conventional epufluorescence light microscopy in that it eliminates the emission coming from above or below the plane of focus. Through this improvement of image quality, the higher resolution achieved enables the detection of considerably smaller structural details within the thickness of biological specimen. It obviously does not offer the resolution of electron microscopy but still permits through the use of thin optical sectioning the identification of cellular organelles with a resolution of approximatly 0,2μm. Finally, the coupling of confocal microscopy with a computer/image processing system not only offers an effective increase in imaging capability but it also vastly increases the possibilities for specimen analysis and presentation.

Of particular interest with regard to the present work is the enhanced resolution and sensitivity of the detection of antibodies conjugated to different fluorescent dyes such as fluorescein isothiocyanate (FITC) and Texas Red. This property becomes particularly critical for the visualization of dually labeled immunocytochemical preparations in which the amount of information that can be obtained using conventional epifluorescence technique is limited by the background fluorescence. Indeed, confocal microscopy suppresses quite effectively the out-of-focus contributions and enables simultaneous detection of more than one fluorophore during the same scanning session. Given that the

specimen under study is double-labeled with each component having its respective excitation wavelength, it is possible to scan each fluorophore at a particular plane of focus. The merging of both confocal images generates an image which enables the analysis of the relative distribution of the markers. Thus, by means of multiple detectors, adequate beam splitting mirrors and filters it is possible to observe in a neuron the coexistence of two different neuropeptides detected via two different fluorophores.

Specific Aims

The specific objectives of this project are to study different aspects of the NT/NN biosynthetic pathway by: (1) localizing the NT/NN precursor derivatives synthesized in the rat CNS; (2) mapping the neuronal populations expressing those derivatives in order to appreciate their overlap and determine if there is differential processing of the precursor between main regions; (3) making an evaluation of where the maturation is taking place by comparing the relative densities of immunolabeling for the different maturation products at the level of the cell bodies and nerve terminals; (4) determining if the different derivatives are in the same vesicles to help establish the intracellular location of post-translational processing; and finally (5) determining which enzymes are involved in the processing of the precursor.

To these ends, it was necessary to generate antibodies against specific regions of the precursor. These antibodies were synthesized in the laboratory of Dr. Kitabgi (Nice) and directed against regions of the precursor molecule adjacent to the presumptive sites

of cleavage. In a first set of experiments, these region specific antibodies were employed, after characterization, in an immunohistochemical investigation designed to identify the neuronal populations containing different NT/NN precursor products and to compare their topographic and cellular distribution. In a second set of experiments, the same antibodies were employed for a high resolution immunocytochemical analysis of NT/NN neurons to determine if the different derivatives of pre-proNT are stored in the same or in distinct neuronal populations (differential processing) and to determine whether they are stored in the same subcellular compartments.

For the first set of studies, each antibody was visualized using the peroxidase anti-peroxidase (PAP) technique on serial sections. For the second series of experiments, they were visualized by fluorescence immunocytochemistry in the same sections in order to confirm their presence within the same neurons and compare their sub-cellular distribution. The analysis of the latter preparations was carried out using confocal microscopy which facilitates the simultaneous visualization of two different markers and provides higher resolution than conventional epifluorescence for the sub-cellular localization of the antigens.

In a third series of experiments, we attempted to determine whether the protein convertases PC1 and PC2 might be involved in the processing of the NT/NN precursor. For this purpose, the distribution of PC1 and PC2 immunoreactivity was compared to that of NT using an immunocytochemical amplification technique. The antibodies directed against the PC1 and PC2 enzymes were synthesized and characterized in the laboratory of Dr. Seidah (Montreal).

EXPERIMENTAL PROCEDURES

Antibodies

Neurotensin (NT) antisera raised in rabbit (for single labeling experiments) and rat (for double labeling studies) were purchased from Incstar and Eugene Tech., respectively. Antibodies against the exposed KLPLVL (K6L) and EKEEVI (E6I) sequences of the NT/NN precursor were raised and characterized as described (Bidard and others 1993). They are selectively directed against the free N-terminal (K6L) and C-terminal (E6I) extremities of the corresponding peptides (Fig 5.). Native NT and native neuromedin N (NN) were purchased from Sigma. The peptides KLPLVLY (K7Y) and YEKEEVI (Y7I) were custom synthesized by Neosystem (Strasbourg, France). For the detection of the maturation enzymes, the AbC-mPC1 and AbC-mPC2 antibodies were used (Benjannet, 1992). These polyclonal antibodies were raised in rabbit and directed against the C-terminal segment 629-706 of mPC1 (Seidah, 1991) and 529-637 of mPC2 (Seidah, 1990), respectively. Their characteristics were described and their specific recognition of PC1 (87-80 KDa) or PC2 (75-65 KDa) demonstrated (Benjannet, 1992; Seidah, 1992).

Colchicine Administration

Adult male Sprague-Dawley rats (185-200 g) were anaesthetized intraperitoneally with sodium pentobarbital (Somnotol, 80 mg/kg, i.p.), injected stereotaxically with 30 μl of colchicine (2 mg/ml) into the left lateral ventricle according to the coordinates of

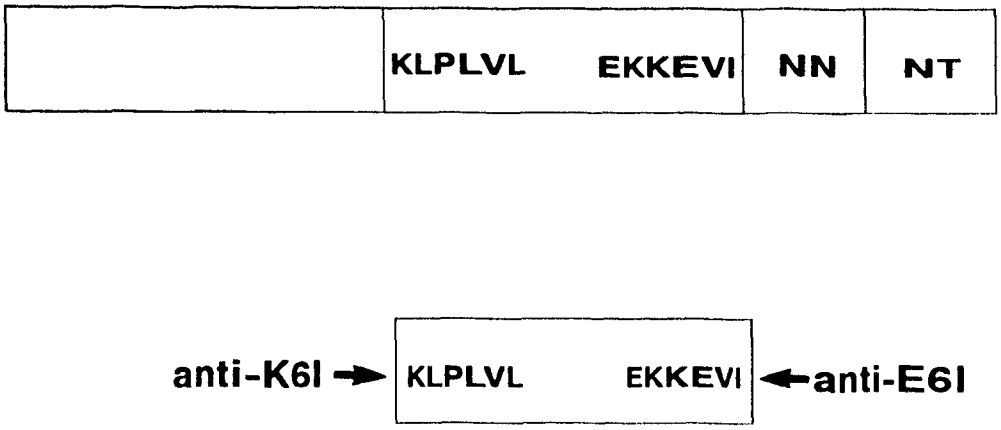


Figure 5. Schematic representation of the NT/NN precursor showing the sequence against which the antisera E6I and K6L are directed. Peptide sequences of interest are written according to the one letter amino acid code, and are derived from the rat precursor sequence.

Paxinos (Paxinos, 1986) (Anterior/posterior: -0,80; lateral/medial: -1,30; dorsal/ventral: -3,7) . The rats were allowed to survive 48 hours before sacrifice.

Tissue Fixation

Untreated (n=8) and colchicine-injected (n=8) rats were perfused transaortically with 500 ml of 4% paraformaldehyde and 0.2% picric acid in 0.1 M Sorensen buffer, pH 7.4. The brains were removed and immersed in the same fixative for 90 min., then stored overnight in a solution of 0.2 M potassium phosphate buffer (PB) containing 30% sucrose. The brains were frozen for 1 min. in liquid isopentane at -50°C.

Immunohistochemistry

NT/NN precursor derivatives

Single labeling experiments

Single labeling studies were carried out according to a modification of the peroxidase anti-peroxidase method of Sternberger (Sternberger 1979). Sets of 3 adjacent 30 μm coronal sections were cut through the brain on a freezing microtome from the level of the genu of the corpus callosum rostrally to the caudal mesencephalon caudally and collected in 0.1 M PB. The sections were then incubated for 20 min. in 0.3% H_2O_2 to quench endogenous peroxidase activity. Following several rinses in 0.1M Tris-buffered saline, pH 7.4 (TBS), the sections were incubated for 30 min in 0.1 M TBS containing 3% normal goat serum (ngs) and incubated for 48 hours at 4°C in primary

antiserum diluted in 0.1 M TBS containing 1% ngs and 0.2% Triton-X 100. One of each of the 3 adjacent sections was incubated in one of the primary antisera against NT (diluted 1:5000), E6I (diluted 1:1500) and K6L (diluted 1:1500). The sections were then rinsed briefly in 0.1 M TBS containing 1% ngs and incubated for 40 min with a goat anti-rabbit immunoglobulin diluted 1:50 in 0.1 M TBS and 1% ngs. Following 2 additional rinses in 0.1 M TBS containing 1% ngs, the sections were incubated for 40 min with a rabbit peroxidase anti-peroxidase complex diluted 1:50 and rinsed again in 0.1 M TBS. Visualization of bound peroxidase was achieved by reaction in a solution of 0.1 M Tris buffer containing 0.05% 3,3' diaminobenzidine and 0.01% H_2O_2. Sections were then rinsed in 0.1 M PB, mounted out of tap water onto gelatin-coated glass slides, air dried, dehydrated in a graded series of ethanols and delipidated in xylene. The mounted sections were then coverslipped and examined under a Leitz Aristoplan.

Double labeling experiments

For double labeling studies, the sections were preincubated as in the single labeling experiments and incubated for 48 hours at 4°C in 0.1 M TBS containing 1% ngs, 0.2% triton X-100, a 1:2000 dilution of rat anti-NT antibody and a 1:1500 dilution of rabbit anti-E6I or anti-K6L antibody. After thorough washes, the sections were incubated for 1 hour at room temperature with a 1:100 dilution of goat anti-rat FITC-labeled IgGs and goat anti-rabbit Texas Red (TR)-labeled IgGs. The sections were washed several times in 0.1 M TBS, mounted on gelatin-coated slides and coverslipped with Aqua-mount (Polysciences, Inc.). The slide-mounted sections were then stored in

light-tight boxes at 4°C prior to light/confocal microscopic examination.

Light microscopic examination was carried out on a Leitz Diaplan fluorescent microscope using FITC- and TR-specific dichroic filters. Confocal microscopic images were acquired, processed and analyzed using an inverted Leica Diaplan microscope coupled to a 2-50mW argon-ion laser and linked to a MC 68020/68881 computer system equiped with a VME bus and a Leica CLSM imaging software. The laser was set at 488nm for excitation of FITC and TR. The emission filter was a highpass from 550nm. For the analysis of colocalization, scanning was made at 1.0 electronic zoom so that at a magnification of 40x (using a fluotar oil immersion objective) the final {x,y} resolution was of 1 pixel per 0.245μm. Final images were derived from 60 consecutive 0.25 μm-thick optical sections for a total thickness of 15 μm and were scanned for each fluorophore (FITC/TR). From this data, both separate or single merged images of each field were generated using extended focus image reconstruction.

For the analysis of the subcellular distribution of the markers, scanning was made at 2,0 and 4.0 electronic zoom so that at a magnification of 100x (using a fluotar oil immersion odjective) the final {x,y} resolution was of 1 pixel for 0,55μm and 0,024μm, respectively. The final images were derived from 3 x 0,5 of 1,0μm-thick optical sections acquired in series and scanned for each flurophore. The merged images were generated by the combination of the image processied for the FITC fluorophore, with its corresponding image processed for the Texas Red fluorophore. All images were stored on an optical disk and printed using a Focus camera system.

Maturation enzymes

Combined immunocytochemical detection of NT, PC1 and PC2 was carried out using the Blast-HRP™ Blotting amplification system (Dupont) and the ABC-peroxidase standard Kit (Vector). Sets of 3 adjacent 30μm coronal sections were cut on a freezing microtome through the brains of control and colchicine treated animals from the level of the genu of the corpus callosum rostrally to the caudal mesencephalon. One of each of three adjacent sections were labeled as for the single labeling experiments described above with one of the primary antisera against NT (1:20 000), PC1 (AbC-PC1, 1:800) and PC2 (AbC-mPC2, 1:1000).

Following overnight incubation in the primary antiserum, the sections were briefly rinsed in 0.1 M TBS containing 1% ngs and incubated 45 minutes with a biotinylated goat-anti rabbit IgG diluted 1:100 in 0.1 M TBS. The sections were then rinsed twice in TBS and incubated for 1 hour in an Avidin-biotinylated-Peroxidase complex (ABC) according to the manufacturer specifications. After additional rinces, the sections were incubated in a 0.01% biotin-tyramine complex, activated with 0.01% H_2O_2 just prior incubation. The sections were washed several times in 0.1 M TBS before being incubated for a second time in the ABC complex. Visualization of the bound peroxidase was achieved by reaction in a solution of 0.1 M Tris buffer containing 0.05% 3,3'diaminobenzidine, 0.04% Nickel Chloride and 0.01% H_2O_2. Sections were rinsed in 0.1 M PB and immediately mounted onto gelatin-coated slides, air dried, and delipidated in xylene before being coverslipped. The examination was carried out on a Leitz Aristoplan microscope.

Immunohistochemical Controls

Preabsorption of either of the two diluted NT antisera with 10 and 100 μM native peptide abolished NT immunostaining. Similarly, preabsorption of the diluted E6I and K6L antisera with 10 and 100 μM synthetic K7Y and Y7I peptides abolished the immunolabeling for E6I and K6L, respectively. In light of the sequence homology displayed between the primary sequences of neuromedin N and K6L (Kislauskis and others 1988), the K6L antiserum was also preabsorbed with 10 and 100 μM native NN (Sigma). This strategy did not influence the K6L immunostaining observed in the present study.

In double labeling experiments, additional controls were carried out whereby each primary antibody was tested alone using a non-corresponding IgG. Thus, the rat anti-NT was revealed using a goat anti-rabbit IgG and rabbit E6I and K6L antibodies were visualized using goat anti-rat IgGs. No immunofluorescence was detected under either of these staining conditions.

AbC-mPC1 and AbC-mPC2 antisera immunostaining was abolished in the presence of an excess of mPC1 (segment 629-729) and mPC2 (segment 529-637) antigens, respectively. Knowing that the antiserum were raised against a hybrid protein consisting of the mPC1 or mPC2 sequences fused to the C-terminus of the bacterial enzyme glutathione-S-transferase (Benjannet and others 1992), experiments were also carried out in which the antibodies were incubated with an excess of this protein. The immunostaining for the AbC-mPC1 and AbC-mPC2 antibodies was not affected by this preincubation.

RESULTS

NT/NN precursor maturation products

Single labeling studies

Normal rats

Light microscopic examination of adjacent sections processed for the immunohistochemical detection of neurotensin (NT), the C-terminal extremity of the Glu^{134}-Ile^{139} (E6I) and the N-terminal extremity of the Lys^{87}-Leu^{92} (K6L) sequences of the precursor revealed immunolabeling of neuronal perikarya, dendrites, axons and axon terminals for all three antigens. These immunostained structures exhibited a heterogeneous topographical localization throughout the brain.

In sections from normal, untreated rats, immunoreactivity for NT, E6I, and K6L was confined almost exclusively to axons and axon terminals (Figs. 6-8). A few, lightly stained neuronal cell bodies were detected for NT and E6I antigens in the lateral septum, bed nucleus of the stria terminalis, central (Fig. 8) and medial amygdaloid nuclei and mediodorsal thalamic nucleus. Only sparse, weakly labeled K6L-immunoreactive perikarya were visible within the bed nucleus of the stria terminalis and the central nucleus of the amygdala.

E6I terminal immunoreactivity was comparable to that observed for NT with respect to both its topographic pattern of distribution and its relative density of immunostained varicosities. Thus, in the forebrain, NT and E6I terminal immunolabeling was prevalent in the nucleus accumbens (Fig. 6), bed nucleus of the stria

terminalis and lateral septum. Dense terminal immunostaining was also noted in the olfactory tubercle and substantia inominata (Fig. 6), as well as along the dorsal and medial boundary of the globus pallidus.

In the hypothalamus, NT and E6I immunolabeled terminals were concentrated in the lateral and dorsal hypothalamic areas, perifornical region, paraventricular nucleus and external zone of the median eminence. Terminal immunolabeling for NT and E6I was also prominent in the zona incerta, medial forebrain bundle and ansa lenticularis. Further caudally, NT and E6I immunoreactivity was detected in the supramammillary region and the mammillary bodies. Axons immunoreactive for NT and E6I were identified coursing through the stria terminalis (Fig. 7a, b). In this fibre bundle, however, NT immunostaining appeared slightly more intense than that displayed by E6I (Fig. 7a, b).

In the thalamus, NT- and E6I-immunolabeled terminals were detected in the mediodorsal, paraventricular, and paracentral thalamic nuclei. The immunostained fibres in these dorsomedial nuclei formed a thin rim around the paratenial nucleus. In addition, terminal labeling was observed in the medial and lateral habenular nuclei. The densest enrichment of NT- and E6I-immunoreactive terminals in the thalamus was detected immediately dorsal to the medial lemniscus, in the region of the medial ventroposteromedial and gustatory nuclei and, further caudally, in the subparafascicular nucleus. The central nucleus of the amygdala, and most notably its medial subdivision, exhibited the densest accumulation of NT- and E6I-immunoreactive varicosities in the brain (Fig. 8). In the cerebral cortex, the most conspicuous immunolabeling for both NT

and E6I antigens was localized to a distinct band of small varicosities in the cingulate, retrosplenial and perirhinal areas.

In the mesencephalon, NT- and E6I-immunoreactive axon terminals and preterminal processes were identified throughout the substantia nigra pars compacta and pars lateralis. Both the paranigral and parabrachial pigmented subdivisions of the ventral tegmental area, as well as the interfascicular nucleus and retrorubral field were densely innervated by NT- and E6I-containing axon terminals. The rostral and caudal linear raphe nuclei, dorsal raphe nucleus, central grey, and marginal zone of the medial geniculate nucleus all displayed moderate densities of NT- and E6I-immunopositive axonal varicosities.

K6L terminal immunostaining was also detected in most of these regions, but was consistently less intense than that displayed by NT and E6I (e.g., Figs. 7, 8) except in the globus pallidus where E6I- and K6L-immunoreactive fibres were both more numerous and more intensely labeled than NT- immunostained ones (Fig. 8). The diminished intensity of K6L immunostaining relative to that of NT and E6I was most conspicuous at mesencephalic levels. In fact, K6L immunoreactivity in the midbrain was only detectable in regions which displayed intense NT and E6I terminal immunolabeling, such as the substantia nigra pars lateralis.

Colchicine-treated rats

Intracerebroventricular colchicine pretreatment substantially enhanced the number

and labeling density of NT-immunopositive cell bodies throughout the brain. E6I-immunoreactive neuronal perikarya were also more numerous and more intensely labeled than in sections from non colchicine-treated rats but remained consistently less numerous than those immunoreactive for NT (Figs. 9, 10). Colchicine treatment had little effect on K6L perikaryal immunoreactivity which remained weak throughout (Figs. 9, 10).

In the forebrain of colchicine-treated rats, NT- and E6I-immunoreactive perikarya were prevalent in the lateral septum and were co-extensive ventrally with a very dense accumulation of immunolabeled cell bodies in the bed nucleus of the stria terminalis (Fig. 9). Somata immunoreactive for these two antigens were also detected along the medial border of the caudate putamen, immediately adjacent to the lateral ventricle. In the nucleus accumbens, NT- and E6I-immunopositive perikarya surrounded the anterior limb of the anterior commissure. The ventral pallidum and medial forebrain bundle also exhibited numerous NT- and E6I-immunolabeled cell bodies. A few NT- and E6I-containing perikarya were detected in the horizontal limb of the diagonal band of Broca. Further caudally, NT- and E6I-immunoreactive neurons were observed in the medial preoptic nucleus, lateral preoptic area, and rostral preoptic periventricular nucleus. In addition, immunostained perikarya extended dorsally from the bed nucleus of the stria terminalis towards, and into, the stria terminalis itself. Within these forebrain regions, cell bodies immunoreactive for K6L were sparsely distributed within the lateral septum, bed nucleus of the stria terminalis (Fig. 9), horizontal limb of the diagonal band and lateral preoptic area.

Within the hypothalamus, NT- and E6I-immunolabeled neurons were most

prominent in the periventricular, parvocellular subdivision of the paraventricular, and arcuate nuclei. Perikarya immunoreactive for NT and E6I were also evident throughout the lateral hypothalamus and perifornical region. A few were also identified within the retrochiasmatic area. Here again, only sparse K6L-immunoreactive neurons were detected, all of which were confined to the lateral hypothalamus.

In the thalamus, NT-, E6I- and a few K6L-immunoreactive neurons were detected in the ventroposteromedial thalamic nucleus, immediately dorsal to the medial lemniscus. In addition, the paraventricular and precommissural thalamic nuclei exhibited NT- and E6I-immunostained cells but were relatively devoid of K6L-immunopositive perikarya. The amygdala displayed a conspicuous accumulation of NT-immunoreactive cell bodies which were concentrated within the medial aspect of the central nucleus (Fig. 10). Although substantially fewer in number, E6I- and K6L-immunoreactive cell bodies were also detected in the central nucleus of the amygdala (Fig. 10). In addition, NT-, E6I- and a few K6L-immunopositive neurons were sparsely dispersed within the medial amygdaloid nucleus. Surprisingly, E6I- and K6L-immunoreactive cell bodies, but not NT-immunoreactive ones, were detected within the granule cell layer of the hippocampal dentate gyrus.

In the ventral mesencephalon, NT- and E6I-immunoreactive perikarya were detected within the ventral tegmental area, substantia nigra pars lateralis and the caudal linear raphe nucleus. A few isolated NT- and E6I-immunolabeled neurons were also observed in the dorsolateral aspect of the substantia nigra pars compacta. In the central grey, NT- and E6I-immunostained perikarya occupied a position dorsolateral to the

cerebral aqueduct, immediately ventral to the posterior commissure. K6L-immunostained perikarya in the midbrain were sparse and confined to the ventral tegmental area.

Colchicine pretreatment substantially decreased the intensity of NT terminal immunostaining in virtually all areas where it had been detected in untreated rats (compare Figs. 8a and 10a). E6I terminal immunoreactivity was even more affected than that of NT by colchicine pretreatment. In fact, whereas in control animals the intensity of E6I immunostaining in axon terminals was comparable to that of NT, in colchicine-treated rats it was consistently less marked (Figs. 10, 11) except in the globus pallidus where, as in untreated animals, E6I immunostaining was both more robust and more widespread than that of NT (Fig. 11a, b). Similarly, K6L terminal immunostaining was markedly diminished in colchicine-treated as compared to untreated animals (compare Figs. 8c and 10c), except in the globus pallidus where it was almost as dense and extensive as that of E6I and hence considerably more pronounced than that of NT (Fig. 11). In all other regions of colchicine-treated rats, K6L-immunoreactive axons were sparse and only faintly labeled (Fig. 11c).

Fig. 6. Darkfield photomicrographs comparing the distributional pattern and intensity of NT (a) and E6I (b) immunostaining in adjacent sections through the nucleus accumbens (Acb) an substantia inominata (SI) of an untreated rat. A dense network of thin varicose axons uniformly pervades the two structures. Note the similarity between NT and E6I immunolabeling patterns. Abbreviation: ac, anterior commissure. Scale bar: 100 μm.

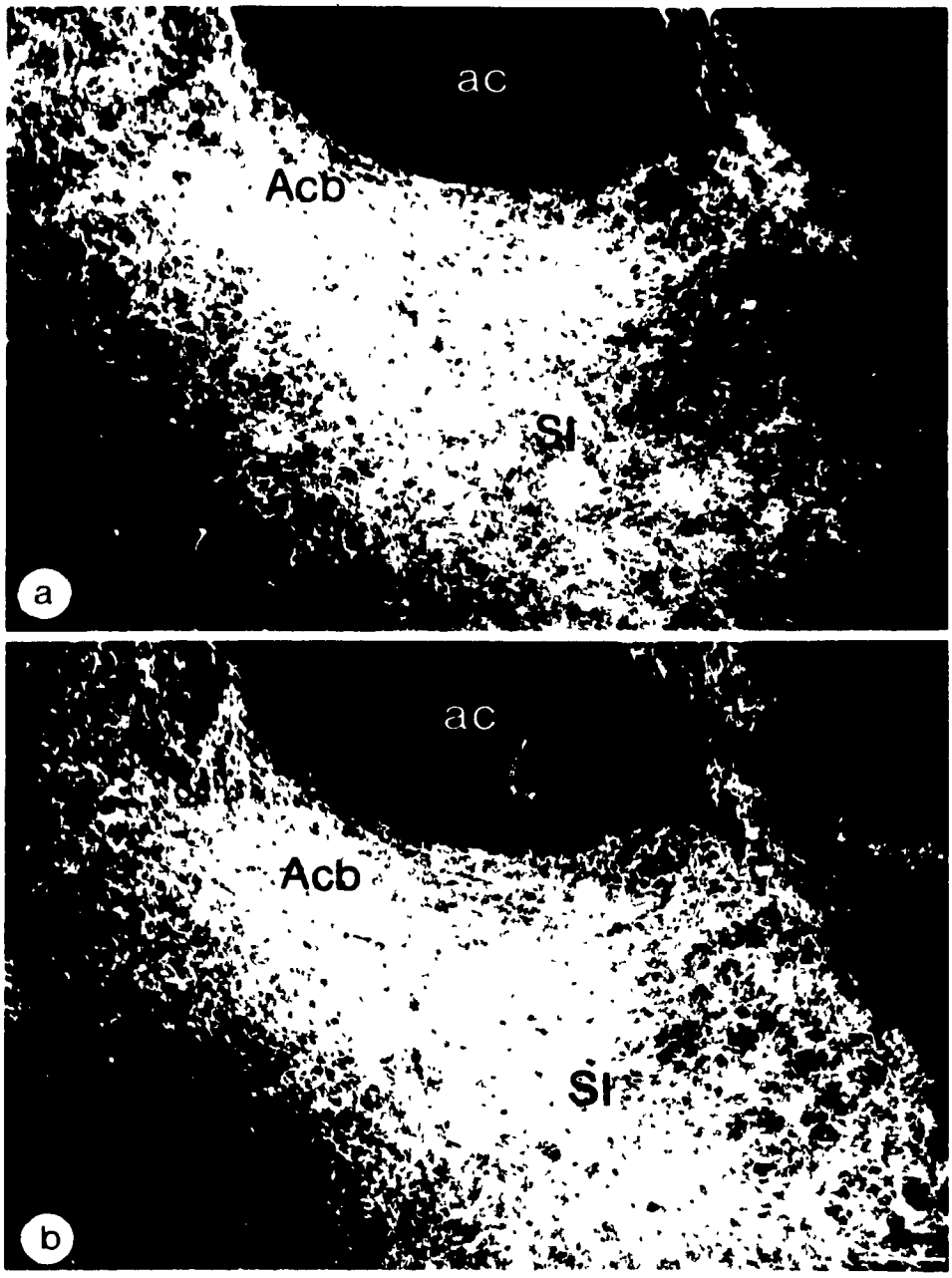

ac
Acb
Sl
a
ac
Acb
Sl
b

Fig. 7. Darkfield photomicrographs of adjacent sections through the stria terminalis (st) and globus pallidus (GP) of an untreated rat. The sections were processed immunohistochemically for the localization of NT (a), E6I (b) and K6L (c). NT immunolabeling of axons coursing through the stria terminalis appears more robust than that observed for E6I. Only sparse K6L-immunoreactive axons are visible within the same structure. By contrast, terminal labeling in the globus pallidus is both more intense and more pervasive in E6I and K6L than in NT immunoreacted material. Abbreviations: ic, internal capsule; LV, lateral ventricle. Scale bar: 75 μm.

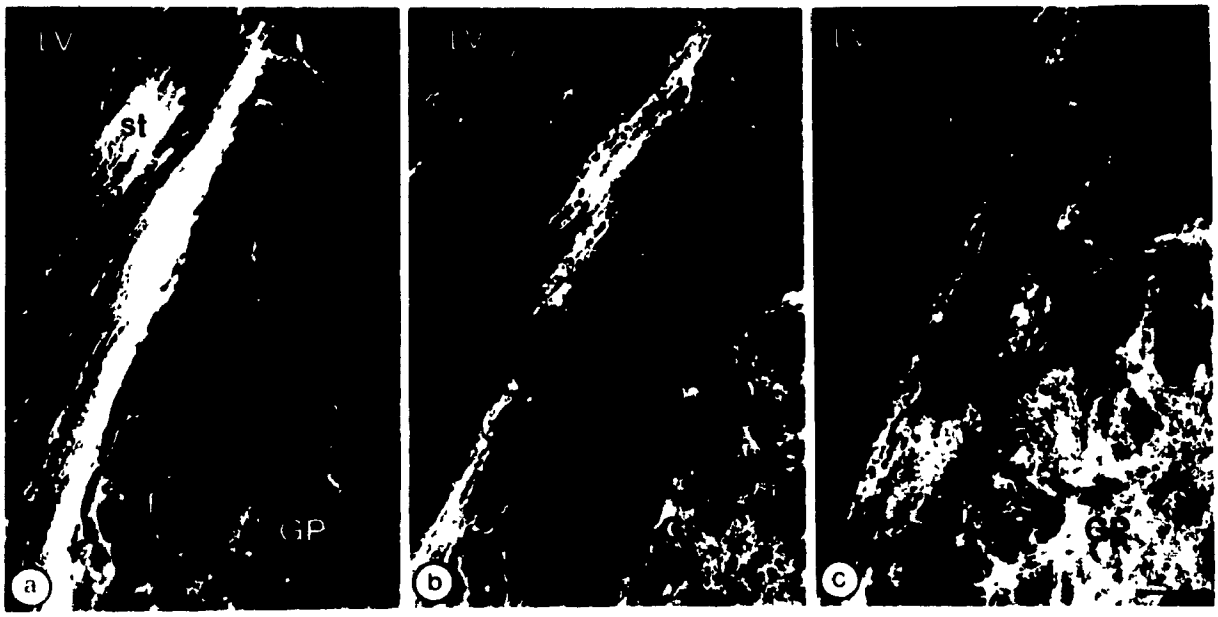

LV
st
GP
a
b
c

Fig. 8. Brightfield photomicrographs illustrating the distribution of NT (a), E6l (b) and K6L (c) immunostaining in the central nucleus of the amygdala (CeA) of an untreated rat. Immunolabeled structures consist almost exclusively of axons and axon terminals. A few labeled perikarya are also visible in NT-immunoreacted material (arrows). Terminal staining is most intense in the capsular division of the nucleus for all three antigens (arrowheads). Abbreviations: CPu, caudoputamen; BlA, basolateral nucleus of the amygdala. Scale bar: 100μm.

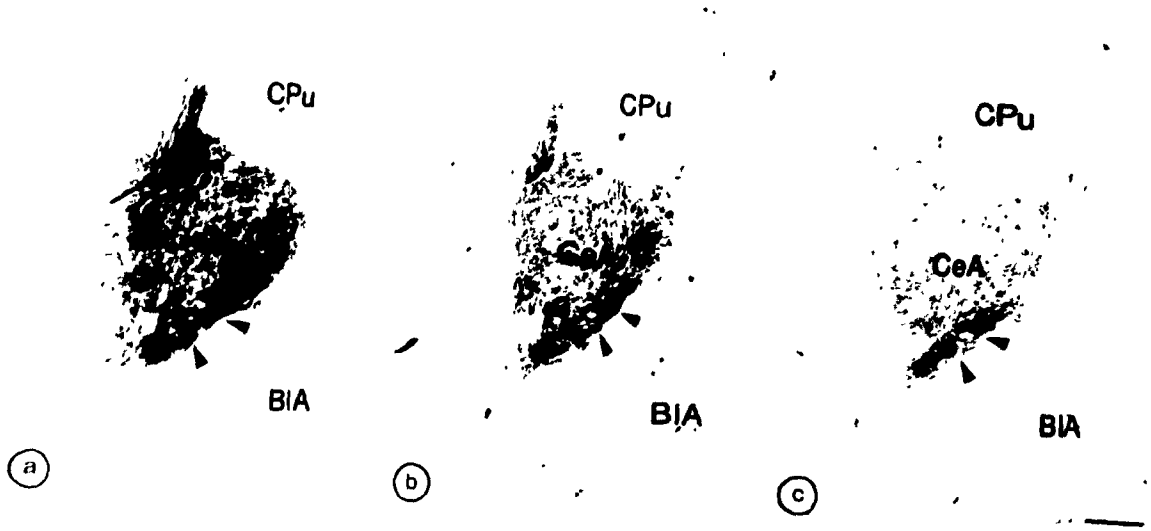

CPu
BIA
a
CPu
BIA
b
CPu
CeA
BIA
c

Fig. 9. NT- (a), E6I- (b) and K6L- (c) immunoreactive cell bodies in adjacent coronal sections through the bed nucleus of the stria terminalis (BST) of a colchicine-pretreated rat. NT-immunoreactive cell bodies are more intensely labeled and far outnumber those displaying E6I immunolabeling. Only a few K6L-immunoreactive perikarya are visible (arrows). Abbreviations: LV, lateral ventricle; CPu, caudoputamen. Scale bar: 75μm.

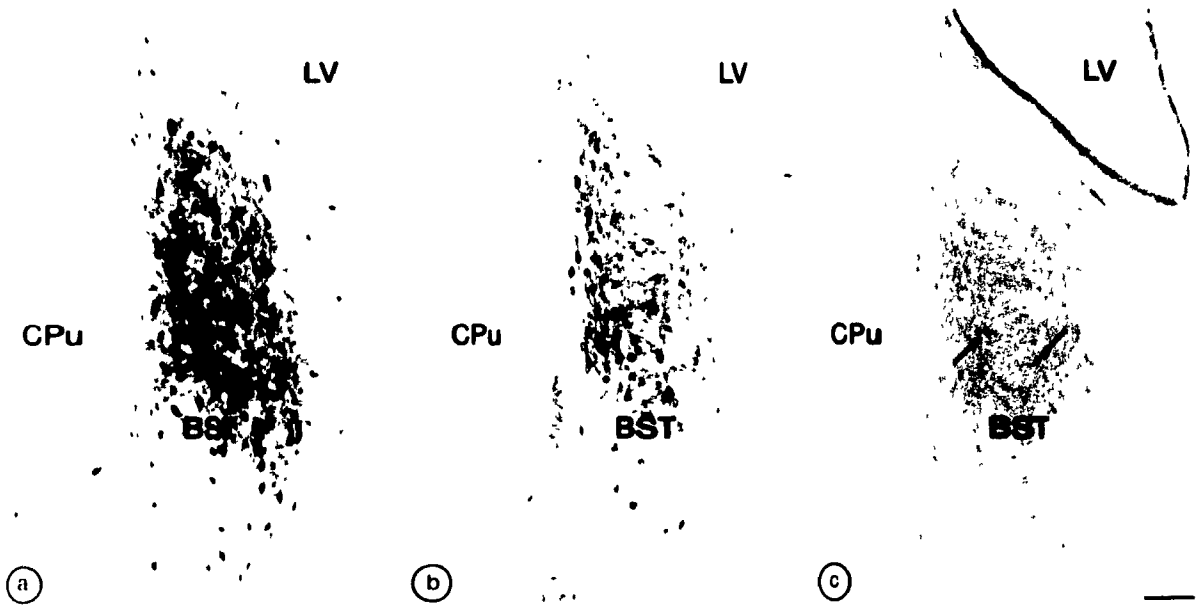

LV
CPu
BST
a
LV
CPu
BST
b
LV
CPu
BST
c

Fig. 10. NT (a), E6I (b) and K6L (c) immunolabeling in adjacent coronal sections through the central nucleus of the amygdala (CeA) of a colchicine-pretreated rat. NT-immunoreactive perikarya and axonal varicosities (arrows) are both more numerous and more intensely labeled than E6I-immunopositive ones. Only sparse, faintly stained perikarya and fibres are apparent in K6L immunoreacted material (c). Abbreviation: CPu, caudoputamen. Scale bar: 50μm

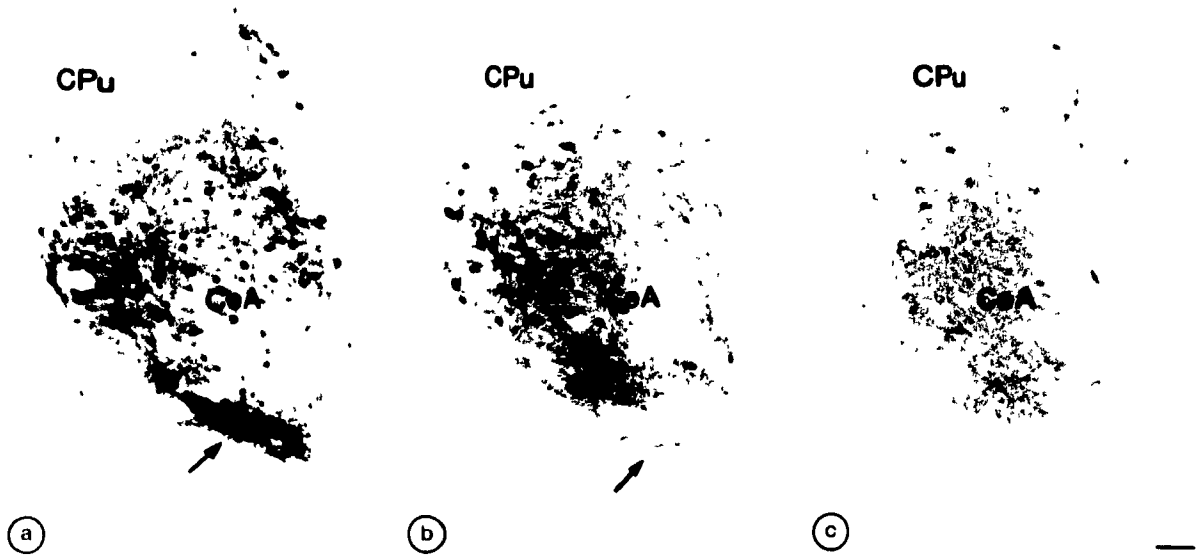
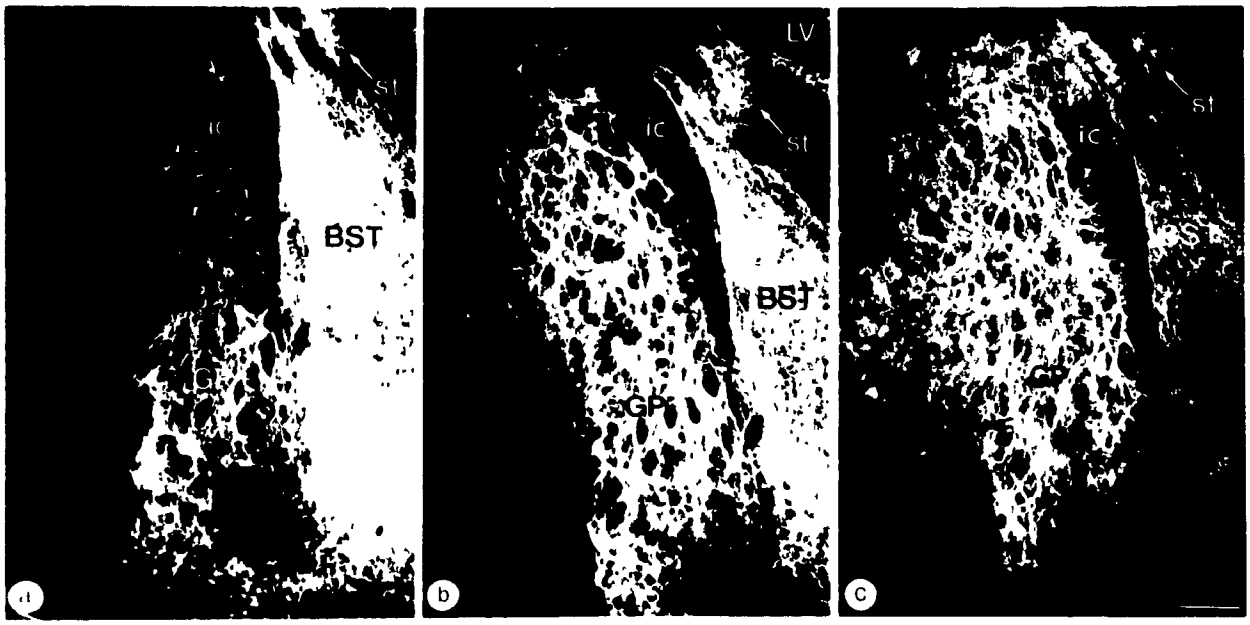

CPu
CoA
CPu
CoA
CPu
CoA
a
b
c

Fig. 11. Darkfield photomicrographs comparing the distribution of NT- (a), E6I- (b) and K6L- (c) immunoreactive fibres in the globus pallidus (GP) and bed nucleus of the stria terminalis (BST) of a colchicine-treated rat. Although the distributional patterns are similar for all three antigens, the labeling densities vary differentially between the two structures such that in the GP, NT < E6I = K6L; whereas in the BST, NT > E6I > K6L. Note the prominent labeling of the stria terminalis (St) in NT- and E6I-immunoreacted sections and its virtual absence in the K6L-reacted material. Abbreviations: LV, lateral ventricle; ic, internal capsule. Scale bar: 200μm.


st
ic
BST
a
LV
ic
st
BST
GP
b
st
ic
c

Double labeling studies

Colocalization of the NT/NN derivatives

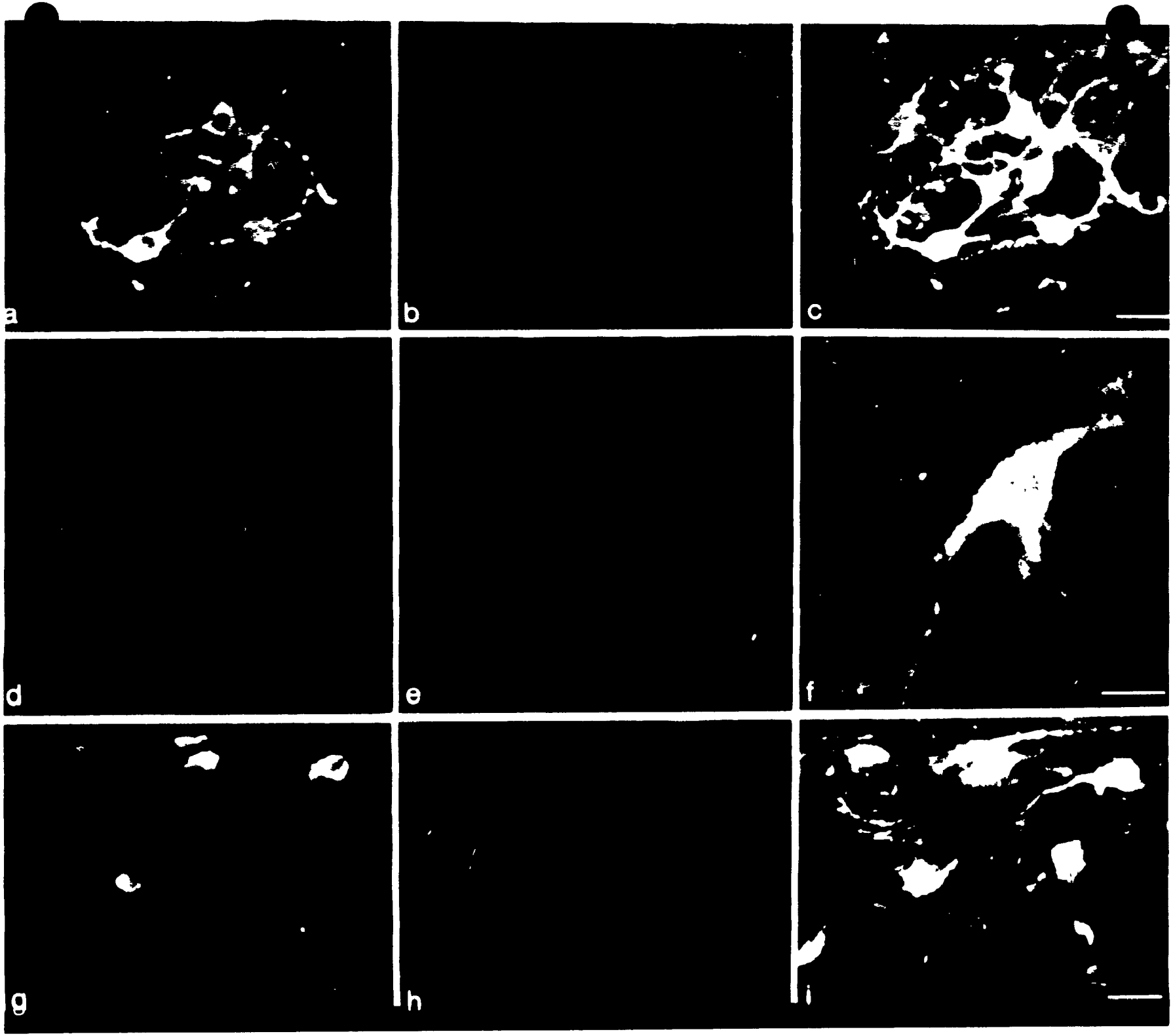
Light microscopic examination of FITC-labeled NT and TR-labeled E6l or K6L immunostaining revealed essentially the same topographic patterns as visualized with the PAP procedure in individually stained adjacent sections. Thus, in normal, untreated rats, only sparse, weakly reactive neuronal cell bodies were detected for all three antigens in the lateral septum, bed nucleus of the stria terminalis, medial aspect of the central amygdaloid nuclei and mediodorsal thalamic nuclei.

In colchicine-treated rats, the number of NT- and E6I- immunoreactive cells was markedly enhanced in all of these regions. In addition, immunoreactive perikarya were detected in the nucleus accumbens, the preoptic and lateral hypothalamic areas and the paraventricular and arcuate nuclei of the hypothalamus. Furthermore, at higher magnification of conventional epifluorescent and confocal microscopes, it was apparent that virtually all E6I-immunoreactive cells were double-labeled, i.e., also showed NT immunoreactivity (Fig. 12a, b; d, e). Confirmation that both antigens were truly in the same cells was achieved by merging individually acquired confocal images (Fig. 12c, f), wherein colocalization is represented by yellow fluorescence.

As in PAP-reacted material, K6L-immunoreactive perikarya remained sparse and weakly labeled throughout, even after colchicine pretreatment. Here again, observation at high magnification of the conventional epifluorescence microscope and by confocal microscopy confirmed that all K6L-immunoreactive cell bodies contained NT-

immunoreactive material (Fig. 12g, h, i).

Fig. 12. a-f: NT (a, d) and E6I (b, e) double-labeled cells in the bed nucleus of the stria terminalis of a colchicine-treated rat; g-i: NT (g) and K6L (h) double-labeled cells in the paraventricular nucleus of the hypothalamus of another colchicine-treated animal. Confocal microscopic images reconstructed from 60 consecutive 0.25 μm-thick optial sections. NT-immunoreactivity (a, d, g) visualized in green (or yellow-green in intensely labeled cells); E6I (b, e) and K6L (h)-immunoreactivity visualized in red. Images on the right (c, f, i) correspond to merged (greed and red) images of corresponding fields. Note that virtually all cells labeled for either E6I or K6L also co-localize NT. Scale bars: a,b,c: 20 μm; d,e,f: 10 μm; g,h,i: 20μm.


a
b
c
d
e
f
g
h
i

Subcellular distribution

The subcellular distribution of the NT/NN precursor maturation products was examined in double labeled material using high resolution confocal microscopy. The analysis was carried out on both normal and colchicine pre-treated animals. As reported above, the colchicine pre-treatment increased both the number and intensity of NT and E6I positive perikarya. However, it did not affect the intracellular distribution of either NT (FITC) or E6I (TR) labeling and hence, the results from both normal and colchicine-treated animals were pooled for presentation. Since there were no K6L immunoreactive cell bodies detectable in normal tissue sections, the information available for this antigen was based solely on the analysis of material from colchicine-treated rats.

Analysis of the intracellular distribution of NT and E6I or K6L immunofluorescent signal was carried out using predetermined acquisition parameters. In a first series of acquisitions, labeled neurons were scanned (step 0,5μm or 1μm) for each marker using either no electronic zooming or a low zooming factor (2). The final images were generated from the integration of multiple optical sections acquired throughout the thickness of the specimen and therefore illustrate the immunofluorescent signal contained in the volume of the neuron. As can be seen in Figs. 13a, b; 14a, b; 15a, b, immunofluorescent signal for NT and E6I was detected in neuronal perikarya, dendrites, axons and axon terminals. The signal for K6L was mostly apparent in axon terminals, although a few weakly labeled perikarya were also detected.

The resolution of the confocal microscope was not sufficient to provide any

information on the subcellular distribution of either of the markers in distal dendrites, axons or axon terminals. However, it was high enough to provide detection of an intracellular compartmentation of the immunofluorescent labeling at the level of the perikarya and proximal dendrites. This compartmentation took the form of intensely fluorescent NT and E6I immunoreactive granules heterogeneously distributed over a low diffuse background (fig.14a, b; 15a, b). In some of the reconstructed images, this background fluorescence was relatively high and found to involve the nucleus (fig.15a, d). In most sections, however, including the single optical sections, it was weak and completely spared the nucleus (Fig.14a; 16a).

To determine whether there was colocalization of the antigens at the subcellular level, a computer algorithm was used to combine in one image the information acquired for each fluorophore. Merging of individually processed images for NT and E6I immunolabeling demonstrated that immunolabeled granules were superimposed, as indicated by the emission of yellow fluorescence (fig. 14c; 15c,f). Owing to the faint fluorescent signal detected with the anti-K6L antibody, the images generated by the merging of individually acquired NT and K6L optical sections were too weak to allow conclusions to be drawn about the colocalization of these two antigens at the level of individual granules. Nevertheless, when we allowed for more light to be reflected onto the detector (through suppression of electronic zooming), it was possible to generate images that confirmed the presence of both antigens in the same cells.

To further characterize the ultracellular distribution of immunofluorescent signal and to confirm that the NT and E6I antigens were indeed colocalized in the same

population of granules, a second set of acquisition parameters was utilized. Double labeled neurons were scanned at the highest magnification using a zooming factor of 4. The final images were generated from the superposition of 3 x 0,5μm-thick optical sections acquired in series from the focal plan at which the surface area was maximal. From these high resolution images (fig.16a, b), we determined that the immunofluorescent granules were heterogeneous in size and shape, and had a diameter varying between 80nm and 200nm. A few larger fluorescent patches were also observed scattered in the cytoplasm which given their irregular shape probably represent fused granular structures. As observed at the lower magnification, the granules were randomly distributed in the cytoplasm of the perikarya, with no preferential accumulation in one region of the cytoplasm over another. Merging of NT and E6I images resulted in a final image displaying a granular pattern of yellow fluorescence, again confirming the colocalization of NT and E6I at the subcellular level. Furthermore, superposition of hand drawings (fig.16c, d) of individually acquired NT and E6I images (fig.16a, b) confirmed that all the granules were double-labeled, i.e. that all the granules detected with the anti-NT antiserum (fig. 16a, c) were also labeled with the anti-E6I antiserum (fig.16b, d).

In summary, the CSLM analysis of the subcellular distribution of the NT/NN precursor derivatives indicates that NT and E6I (hence NN) are mainly concentrated in granular structures heterogeneously distributed throughout the neuron's cell body and that the two antigens are colocalized within the same structures.

Fig. 13. Confocal microscopic images of a neuron double-labeled for NT (a) and E6I (b) in the lateral septum nucleus of a colchicine-treated rat. Micrographs in (a) and (b) are derived from the superposition of 15 x 1μm-thick optical sections acquired with a 40x oil objective. The immunofluorescent signal is distributed throughout the cytoplasm of the perikarya and dendrites. The emisson of yellow fluorescence (c) resulting from the merging of the individually acquired images confirm the colocalization of NT- and E6I-immunoreactivity in the same neuron.

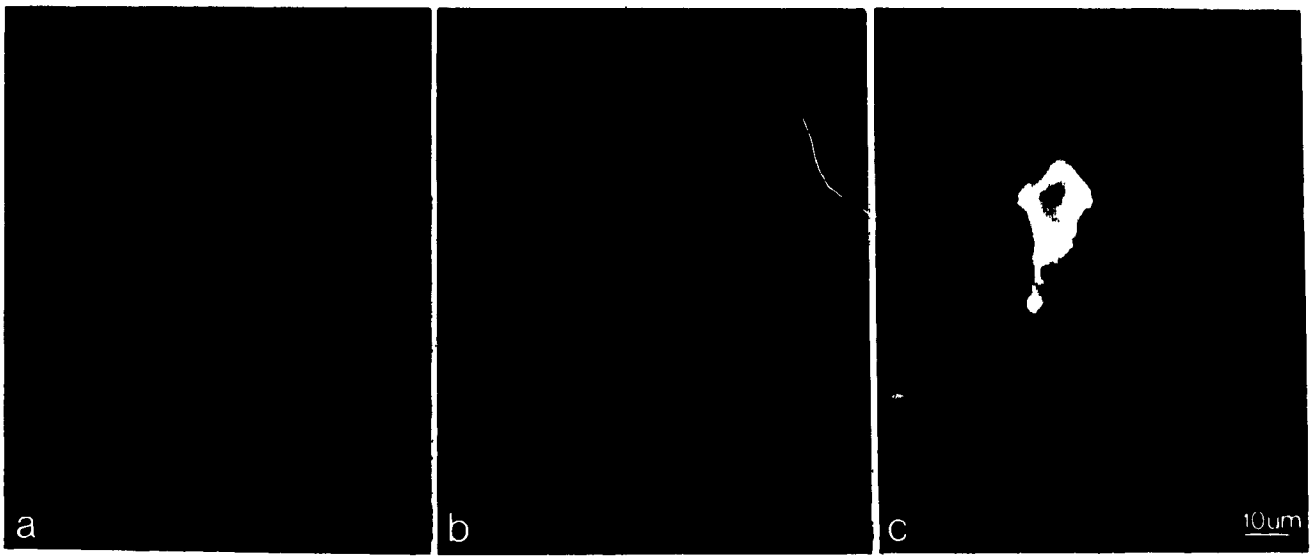
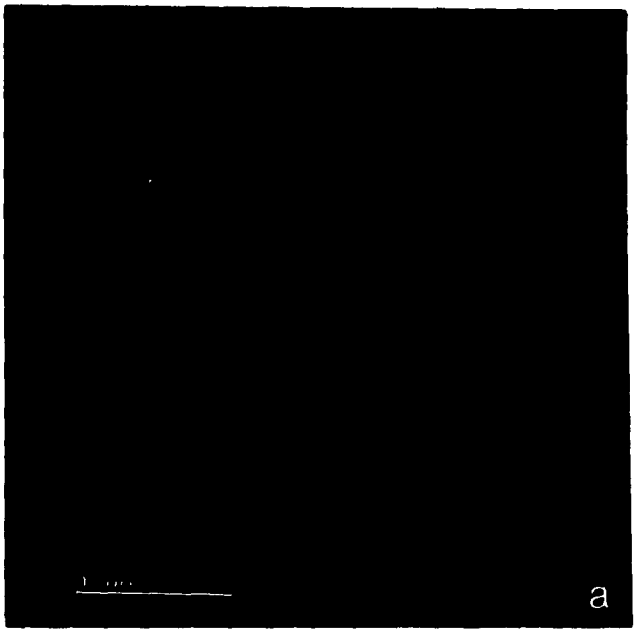
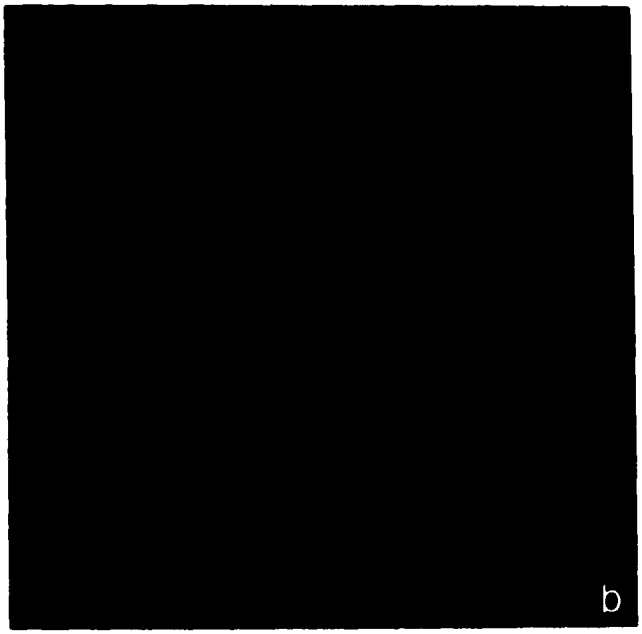

a
b
c
10um

Fig. 14. NT-(a) and E6I-(b) immunoreactive neuron in the bed nucleus of the stria terminalis of a normal rat. Confocal microscopic images reconstructed from 15 consecutive 0,5μm-thick optical sections (optical magnification: x100; zooming factor: 2). Note the granular pattern of NT (FITC) and E6I (Texas Red) labeling throughout the cytoplasm of the perikaryon and the dendrites of the cell. Merging of these individually acquired images generate a yellow fluorescence emission (c) confirming colocalization of NT and E6I antigens within the same subcellular compartments.


a


b

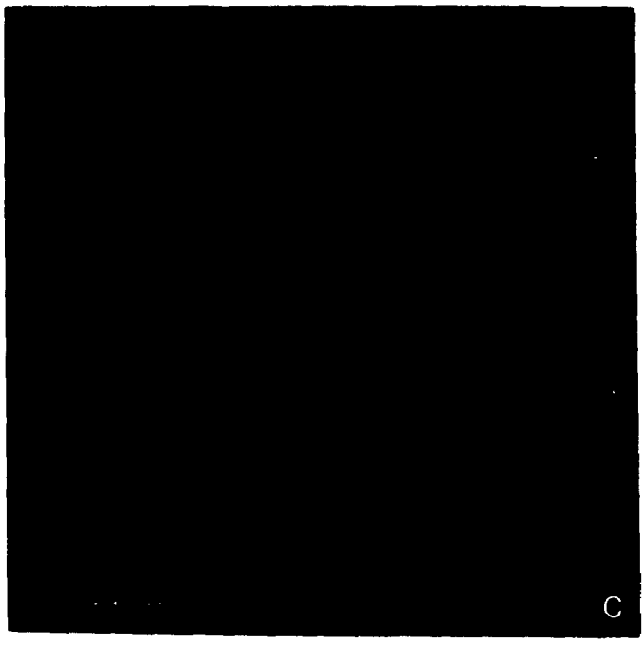

c

Fig. 15. Extended focus images of two neuronal perikarya immunolabeled for NT (a,d) and E6I (b,e) in the arcuate nucleus (a-c) and the central nucleus of the amygdala (d-f) of a colchicine-treated rat. Both series of images were obtained from the two dimensional superimposition of 15 x 0,5μm-thick optical sections acquired in sequence along the microscope optical axis (objective: 100x; zooming factor: 2). Note the granularities of the immunofluorescent signal throughout the cytoplasm of the cell. The emission of yellow fluorescence in panel (c) and (d) results from merging images (green and red) of matching fields and confirms that virtually all granular structures are double labeled.

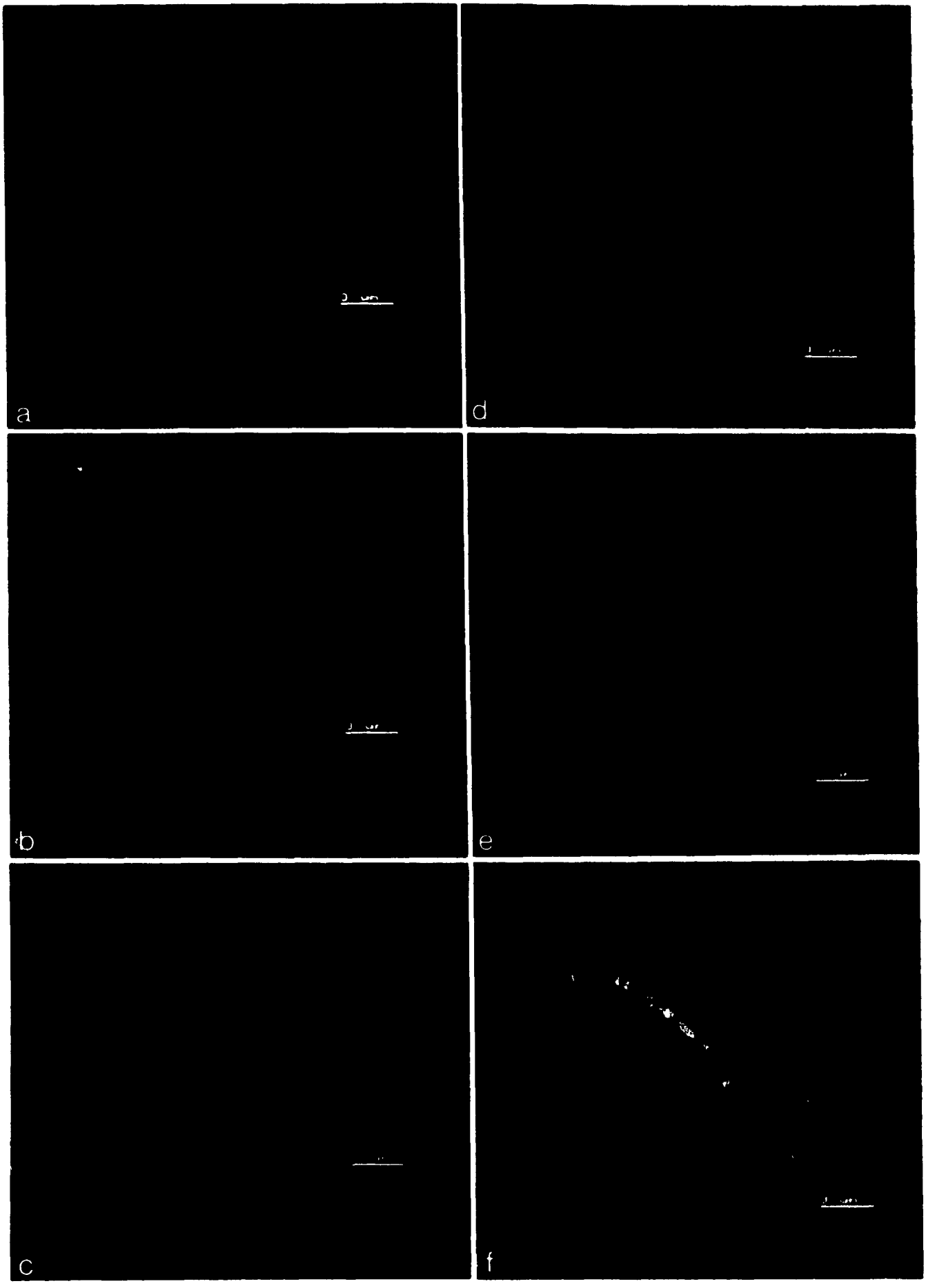
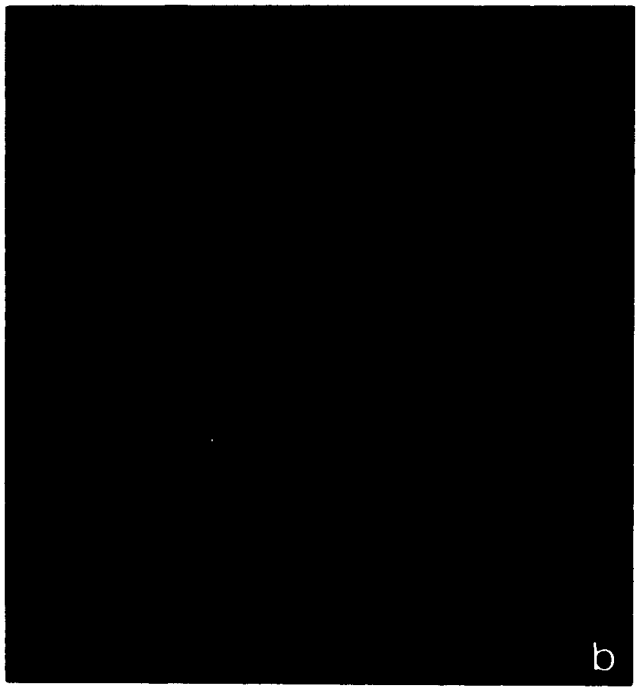
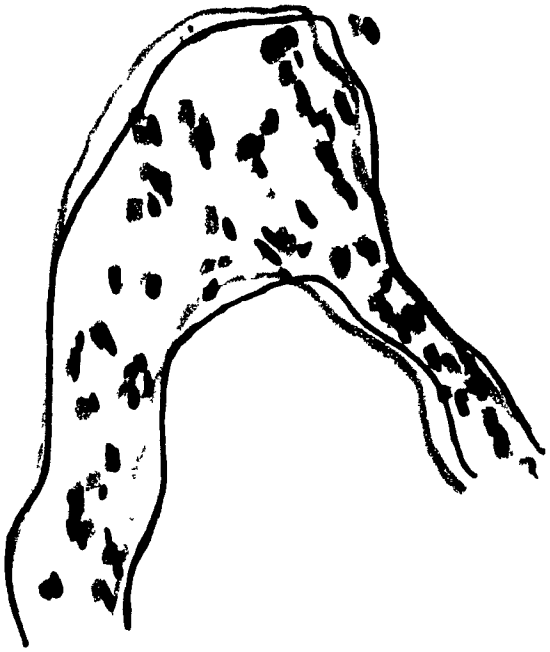

a
d
b
e
c
f

Fig. 16. High magnification (objective: x100; zooming factor: 4) of a dual NT-(a) and E6I-(b) immunoreactive neuron in the central nucleus of the amygdala. The images are reconstructed from only 3 consecutive 0,5μm-thick optical sections. The granular pattern of fluorescence is apparent throughout the cytoplasm of the perikaryon, with the granules varying between 80-200μm in diameter. Hand drawings of panels (a) and (b) are represented in panels (c) and (d), respectively. Superposition of (c) and (d) shows that all the vesicle-like structures detected with the anti-NT (FITC) antibody are also stained with the anti-E6I (TR) antibody , confirming that the NT and E6I antigens are colocalized within the same granules.

(d)





(c)

(d)

Maturation enzymes

An amplification procedure was used here to improve the immunocytochemical detection of the PC1 and PC2 maturation enzymes in rat brain sections. The strong immunolabeling generated by the use of a biotin-tyramine complex enabled the analysis of the brain distribution of PC1 and PC2 relative to that of endogenous NT. However, given that colchicine pre-treatment was necessary for the detection of NT in cell bodies, it was necessary to carry out a preliminary analysis to document whether this treatment affected the distribution of PC1 and/or PC2.

Comparison between sections from normal and colchicine-treated rat processed for the immunocytochemical detection of either PC1 or PC2 revealed no difference in the regional distribution of these enzymes. In both normal and colchicine pre-treated animals, the immunoreactive signal for PC1 and PC2 exhibited an heterogeneous localization throughout the brain with PC2 immunoreactivity being more extensively distributed than PC1 both in terms of labeled structures and density of positive neurons. The PC1 and PC2 signal was confined to cell bodies as well as proximal axons and dendrites except in the internal zone of the median eminence where PC2-immunoreactive terminal fields were observed. Furthermore, there was no apparent difference between control and colchicine-pretreated animals in the number and intensity of PC1 and PC2 immunoreactive signal. We therefore concluded from this preliminary study that brains of colchicine-treated animals could be used to compare the tissue distribution of PC1 or PC2 with that of NT.

The analysis was performed in various areas of the colchicine-treated brain known to express a large amount of NT/NN precursor (as visualized by NT-immunoreactivity). As described in the section on the distribution of the NT/NN precursor derivatives, NT immunoreactive cell bodies were localized within various forebrain regions including the lateral septum, the medial aspect of the caudo-putamen, the preoptic area, the bed nucleus of the stria terminalis, the paraventricular nucleus of the thalamus as well as several nuclei of the hypothalamus. In these selected regions, comparative light microscopic examination of serial adjacent sections processed alternatively for the detection of the PC1, PC2 and NT antigens suggested a partial codistribution of the enzymes with NT at both the regional and cellular levels.

Important regional variations were detected in rostral forebrain regions with regards to the pattern of immunolabeling. In the lateral septal nucleus, numerous NT-positive cells were dispersed throughout the extent of the nucleus, whereas PC2-positive neurons were prevalent in the dorsal subdivision. No PC1-immunoreactive signal was detected in the septal nucleus. At the cellular level, we noted a difference in the organization of NT- and PC2-reactive neurons (Fig. 17). In contrast to the NT-positive neuron which were sparse, the PC2-positive cells were densely packed and had fine projections running parallel to one another.

In the caudoputamen (fig. 18), densely labeled NT perikarya were scattered medially along the dorso-ventral axis of the lateral ventricle (fig.18a). Only few PC1-positive neurons were detected in this structure and they were confined to the same region as the NT-immunoreactive ones (fig. 18b). In contrast to NT- and PC1-positive

neurons, PC2-labeled perikarya were dispersed throughout the striatum where they formed islands or "patches" of the size and distribution of striosomes (fig. 18c).

In the lateral and medial preoptic area, the organization of NT- and PC2-immunoreactive neurons was markedly different. NT-immunoreactive cells were dispersed throughout the region (fig.19a), whereas PC2-immunoreactive cells were both more numerous and more compactly organized (fig. 19c). PC1-immunoreactive neurons were barely detectable and their organization mirrored that of PC2 cells (fig. 19b). At high magnification, a difference in morphology was noted between the NT and PC2 neurons. While NT-positive neurons were triangular in shape and exhibited numerous processes extending in all directions, the PC2-labeled cells were smaller, elongated and exhibited fewer processes.

In the bed nucleus of the stria terminalis (fig. 20a, 21b), NT-immunopositive nerve cell bodies were detected from the dorso-lateral aspect of the anterior commissure rostrally to its ventro-lateral aspect caudally (fig. 20a, 21a). In contrast, strongly labeled PC2 cells were observed only in the antero-medial subdivision of the nucleus (fig.20c), with a few and no labeled neurons in the lateral (fig.21c) and ventral aspects, respectively. Scarce and weakly labeled PC1 neurons were confined to the antero-medial subdivision of the bed nucleus of the stria terminalis (Fig. 20b) in a pattern that displayed no obvious similitude with that of NT-labeling. No particular difference was evident with regard to the morphology of NT, PC1 and PC2-labeled neurons in this structure (fig. 22).

In the paraventricular nucleus of the thalamus, the number, intensity and

distribution of NT-labeled cells was comparable to that of PC1 and PC2-immunoreactive neurons detected in the same region.

In the central nucleus of the amygdala (fig.23), NT-immunoreactive nerve cell bodies were numerous and mainly concentrated in the medial aspect (fig.23a). By contrast, no immunoreactivity was detected within this nucleus with either the anti-PC1 or the anti-PC2 antisera (fig.23b). The same situation was noted in the nucleus accumbens: whereas numerous and strongly-labeled NT neurons surrounded the anterior limb of the anterior commissure, only low to undetectable levels of PC1 and PC2 signal were observed.

In the hypothalamus, densely labeled NT and moderately labeled PC1 and PC2 perikarya were detected throughout the lateral hypothalamus. However, PC1 and PC2 neurons far outnumbered NT ones and also exhibited a more profuse arborization than did NT-immunoreactive cells. Caudally, strongly labeled NT perikarya were prevalent in the periventricular and paraventricular nuclei (medial and parvocellular portions) of the hypothalamus. Moderate PC1 and PC2 immunoreactive signals were detected in the paraventricular nucleus, while low PC1 and undetectable PC2 levels of labeling were found in the periventricular nucleus. It is interesting to note that no NT-immunoreactive neurons were observed in the supraoptic nucleus, where a large number of PC1 and PC2 cells were apparent. Finally, the arcuate nucleus displayed numerous and intensely labeled NT cells, but only a few weakly stained PC1 and PC2 perikarya. The heterogeneous patterns of NT, PC1 and PC2 immunoreactivity in the hypothalamus imply that although the PC1 and PC2 enzymes could be co-localized with NT in some nuclei

(eg. paraventricular), they are unlikely to be colocalized in others (eg. arcuate nucleus).

In sum, the analysis demonstrated that both PC1 and PC2 have an overlapping, yet distinct distribution from that of NT. The codistribution of NT with PC1 and/or PC2 remained partial, despite the fact that the regional overlap of NT with either of these enzymes was considerably more extensive for PC2 than for PC1. At the regional levels, no consistent pattern of immunolabeling (organization, density and intensity) was observed: while comparable in some regions, the pattern of immunoreactivity obviously reflected the presence of distinct populations of cells in other regions.

Fig. 17. High magnification of NT (a) and PC2 (b) labeled neurons in the dorsal subdivision of the lateral septum nucleus of a colchicine-treated animal (adjacent 30μm-thick sections). Note the marked difference in the number and distribution of the neurons labeled with the two antigens. Although numerous NT-terminals are detectable (a) no PC2 terminals are apparent (b). Scale bar: 20μm


a
b

Fig.18. Brighfield photomicrographs comparing the distribution of NT(a), PC1(b) and PC2(c) immunoreactivity (DAB-Ni) in the medial aspect of the caudoputamen of a colchicine treated-rat (adjacent 30μm-thick sections). NT positive neurons (a) are scattered along the dorso-ventral axis of the lateral ventricle. A few PC1-immunoreactive cells of comparable size are detected within the same regions. By contrast, PC2 labeled neurons (c) are dispersed throughout the striatum. LV= lateral ventricle; CPu: caudoputamen; Scale bar: 50μm.

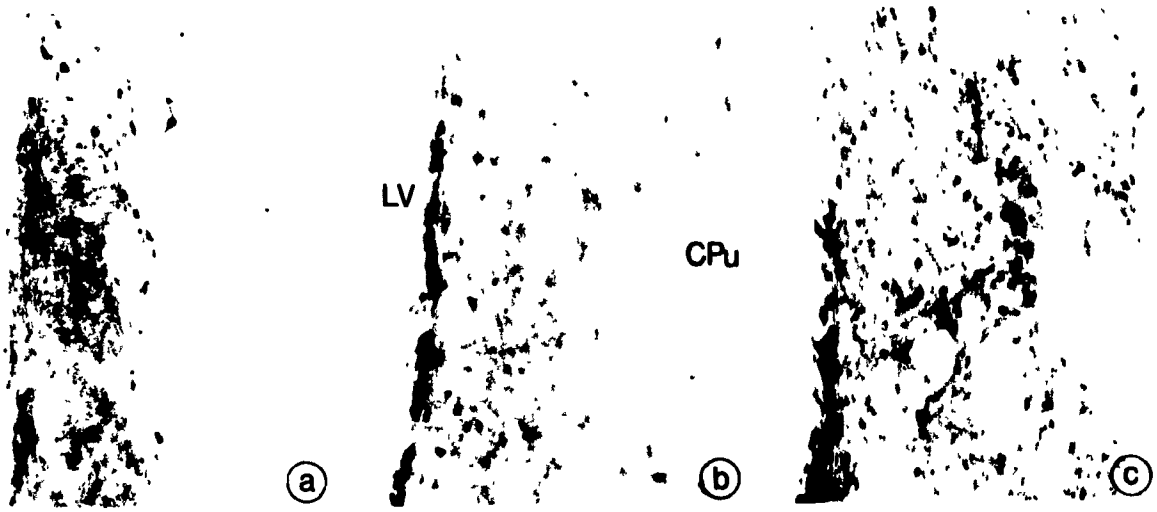

LV
CPu
a
b
c

Fig.19. NT(a), PC1(b) and PC2(c) immunolabeling (DAB-NI) in three adjacent sections taken at the level of the lateral preoptic area in a colchicine-treated animal. Moderately labeled NT neurons (a) are scattered throughout the region. PC1-labeled cells (b)are almost undetectable within the same field. PC2-immunoreactive neurons (c) are much more numerousthan, but comparable in size to, NT-immunoreactive cells. V: third ventricle; ac: anterior commissure; Scale bar : 50μm.

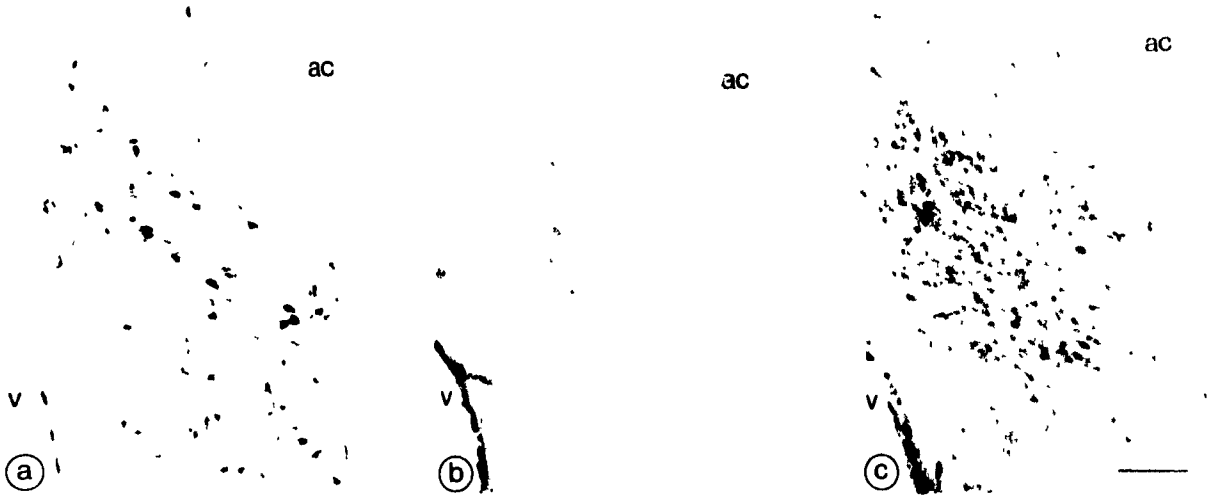

ac
v
a
v
b
ac
ac
v
c

Fig.20. Serial adjacent 30μm-t nick sections from the most anterior aspect of the bed nucleus of the stria terminalis. Here again, PC2-immunoreactive cells (c) are similar in size, but considerably more numerous the NT-immunoreactive ones. By contrast, PC1-immunoreaactive neurons are scarce and show no obvious similitude to the NT labeling pattern. LV: lateral ventricle; CPu: caudoputamen; Scale bar: 50μm.

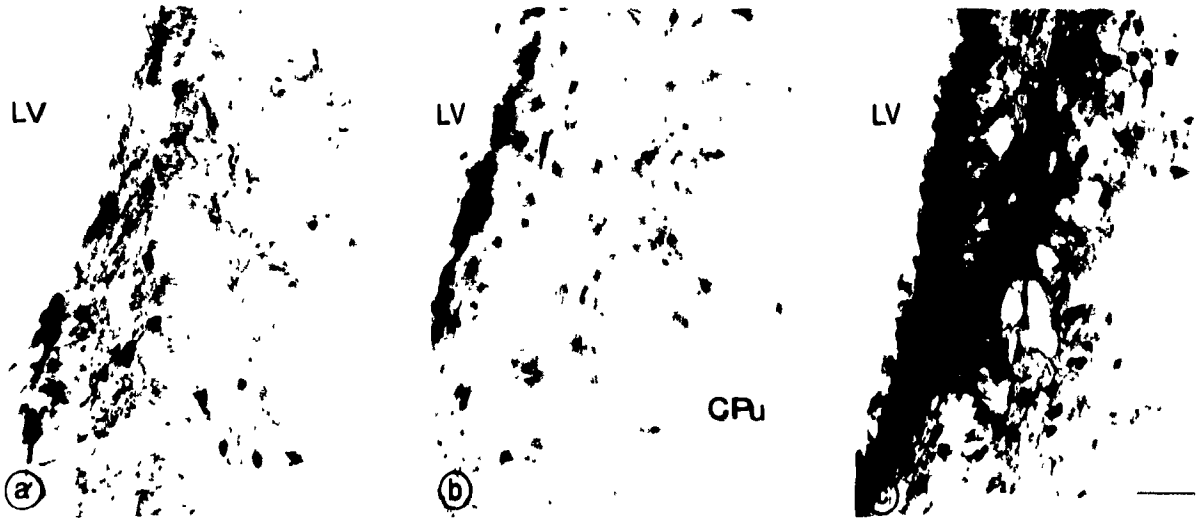

LV
LV
CPu
LV
a
b

Fig.21. Adjacent sections (30μm-thick) through the bed nucleus of the stria terminalis of a colchicine-treated rat. Represented is the amplified immunoreactive signal (DAB-Ni) for NT (a), PC1 (b) and PC2 (c) in the dorso-lateral subdivision of the nucleus. Note the high density of NT positive perikarya as compared to that of PC1- and PC2-labeled neurons. LV: lateral ventricle; BST: bed nucleus of the stria terminalis; ic: internal capsule; Scale bars: 50μm.

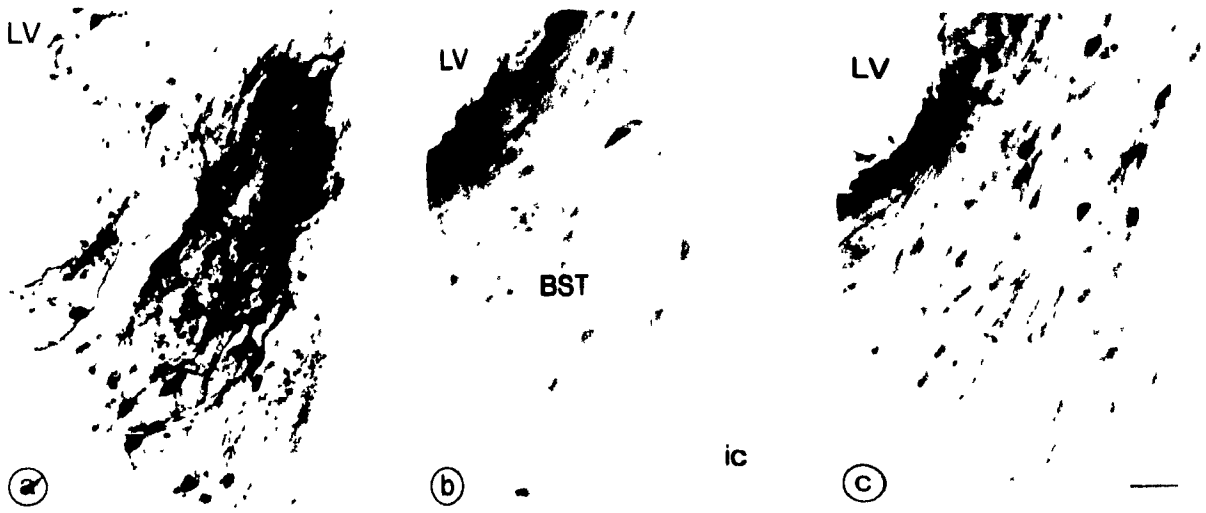

LV
LV
BST
ic
LV
a
b
c

Fig.22. High magnification photomicrographs comparing the NT(a) and PC2 (b) immunoreactivity in the antero-medial subdivision in the bed nucleus of the stria terminalis (adjacent 30μm thick sections; colchicine animal). Note the similarity in size but the difference in density of the two labeled neuronal populations. Scale bar: 20μm

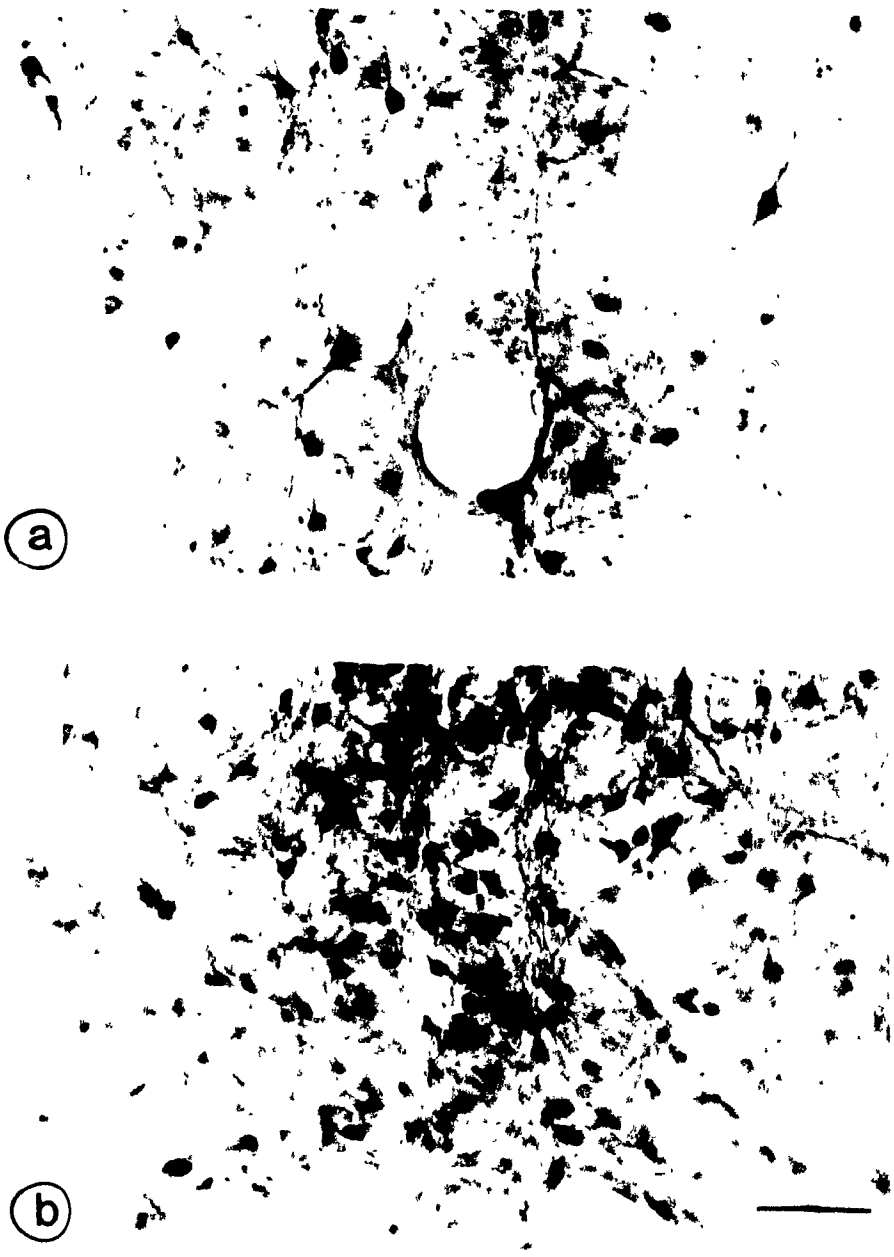
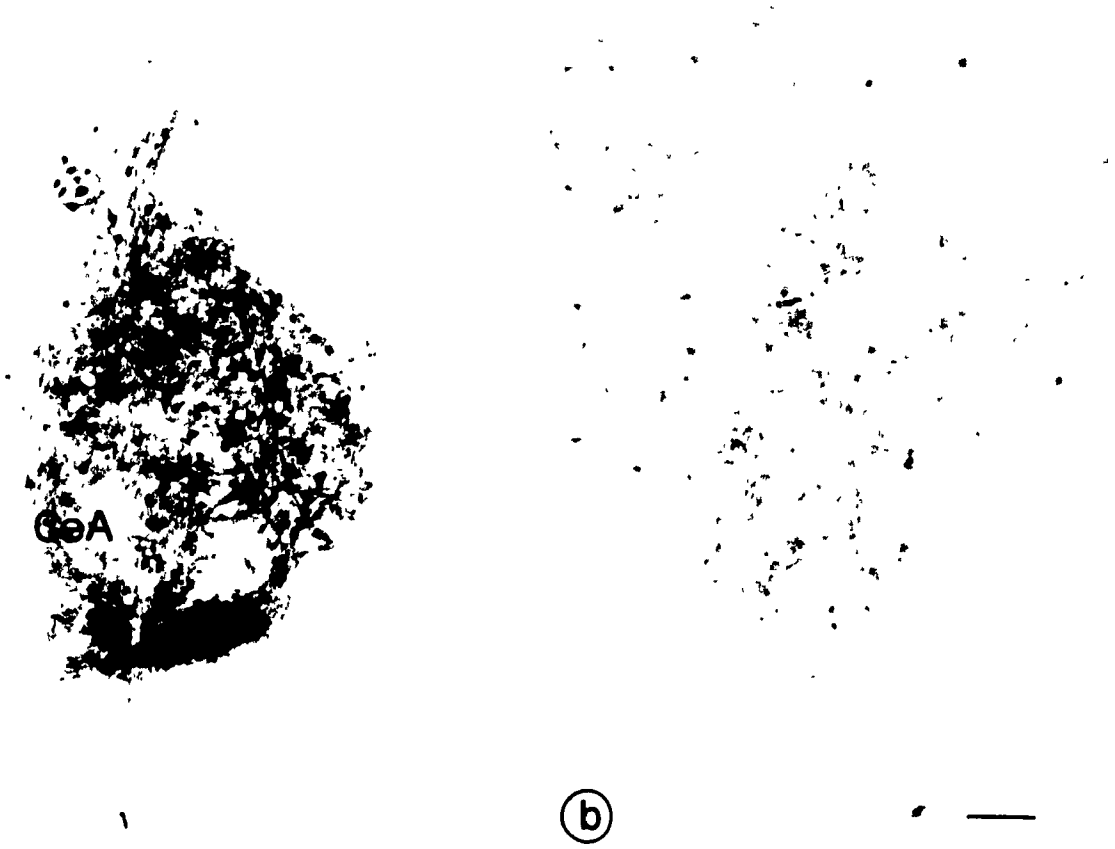

a
b

Fig.23. Adjacent sections (30μm thick) from the central nucleus of the amygdala of a colchicine-treated rat showing the absence of PC2 immunolabeling (b) despite the presence of intense NT (a) immunoreactivity. Scale bar: 75μm.



(a) (b)

DISCUSSION

Distribution of the NT/NN precursor derivatives

The anti-NT antisera employed in the present study were both shown to cross-react specifically with rat native NT (Bayer, 1991a) (de Nadai, unpublished). Pre-absorption of these antisera with synthetic NT completely abolished NT immunostaining, thereby confirming a specific recognition of the peptide. Both antibodies were found to cross-react with the entire NT sequence, although the rat antibody was reported to cross-react most strongly with the 8-13 C-terminal fragment (Bayer, 1991a). Either of these antibodies would therefore have also recognized any N-terminally extended form of the NT molecule, but biochemical studies have shown these forms to account for only a minute proportion of NT immunoreactivity detected in rat brain (DeNadai, 1994). The anti-NT antibodies might also have cross-reacted with the hexapeptide NN, given its considerable sequence homology with the C-terminal portion of NT. However, neither of the two antibodies were found to cross-react significantly with NN (Bayer, 1991a) (de Nadai, unpublished).

The anti-K6L and anti-E6I antisera employed in the present study were generated against the exposed N-terminal and C-terminal extremities of the Lys^{87}-Leu^{92} (KLPLVL; K6L) and the Glu^{134}-Ile^{139} (EKEEVI; E6I) sequences of the NT/NN precursor molecule, respectively (see Fig. 5) (Bayer, 1991a). Consistent with previous biochemical analyses (Bidard, 1993), pre-absorption of these region-directed antisera with the synthetic K7Y and Y7I peptides abolished their respective immunostaining, thereby confirming a specific recognition of endogeneous E6I and K6L sequences by these antisera. Similarly,

despite the considerable sequence homology displayed between the K6L sequence and NN, pre-incubation of the K6L antiserum with native NN failed to produce any apparent diminution of K6L immunostaining in the brain. This is in keeping with the results of biochemical studies which revealed that the K6L antiserum employed in the present study cross-reacted only marginally with NN *in vitro* (Bidard, 1993; DeNadai, 1994). It is thus reasonable to conclude that the K6L immunoreactivity detected in the present study represents precursor derivatives endowed with the 6 amino acid sequence at their N-terminal extremity rather than endogenous NN.

As determined by the biochemical characterization of the NT/NN precursor maturation products, the detection of K6L and E6I immunoreactivity is predicated on cleavage at the dibasic residues flanking the Lys^{87}-Ile^{139} region of the precursor, respectively (DeNadai, 1994). It was inferred from these biochemical data that E6I immunoreactivity mainly corresponds to the Ser^{23}-Ile^{139} segment, i.e., to pro-NT/NN lacking its signal sequence and the NT and NN moieties. It was also postulated on the basis of HPLC migration patterns that K6L immunoreactivity mainly corresponds to the Lys^{87}-Ile^{139} sequence perhaps still linked to the N-terminal part of a larger precursor fragment through a disulfide bridge. However, the possibility that the K6L antibody also detects C-terminally extended forms of the Lys^{87}-Ile^{139} moiety such as Lys^{87}-Leu^{147} (N-terminally extended NN), Lys^{87}-Leu^{162} (N-terminally extended NT) or even Lys^{87}-Tyr^{169} cannot be excluded.

In untreated rats, immunoreactivity for NT, E6I and K6L was almost exclusively confined to axons and axonal varicosities suggesting that the bulk of immunoreactivity

detected in the radioimmunoassays for these antigens is stored in terminal fields (DeNadai, 1994). The regional localization of NT- and E6I-immunoreactive axons in these non-colchicinized animals was similar to that reported previously for NT (Emson, 1985; Jennes, 1982). The intensity of NT and E6I terminal labeling was also comparable for both antigens in all regions examined except in the globus pallidus, as discussed below. This similarity conforms once again with the biochemical data which have shown iE6I to be a major constituent of brain extracts with a concentration of 60-80% of that of NT (DeNadai, 1994) and provides further support for the notion that the Lys^{140}-Arg^{141} doublet between the E6I and NN sequences is a major processing site of the NT/NN precursor in rat brain. The similarity in the distribution of E6I and NT immunoreactivity is also in keeping with previous biochemical evidence for widespread co-processing of NT and NN in rat CNS (Carraway, 1990; Kitabgi, 1991). It would obviously have been of interest to confirm these biochemical findings immunohistochemically through the use of specific anti-NN antibodies. Unfortunately, the antibody that was used in the radioimmunoassay, although applicable to histochemical NN detection in the intestine (de Nadai, unpublished), proved unsuitable for its visualization in rat brain.

Although also confined to regions exhibiting NT and E6I immunoreactivity, K6L immunostaining was consistently much weaker again except in the globus pallidus (see below). This observation is consistent with the biochemical data which have shown K6L immunoreactivity to represent only a small fraction of brain NT contents and confirms that the Lys^{85}-Arg^{86} doublet that precedes the K6L sequence is only partially processed. Nonetheless, the fact that the K6L sequence is exposed at all is in itself worthy of note

since it was not detected in rMTC 6-23 cells (Bidard, 1993) and was found to be only a minor precursor component in rat intestine (deNadai et al., unpublished). It would therefore appear that larger molecular weight intermediates of pro-NT/NN maturation may not be processed to the same extent in other tissues as they are in the brain. Indeed, large molecular weight pro-NT/NN maturation products containing the NN moiety were recently shown to constitute a high proportion of iNN detected in the heart, adrenal and intestine but only a small fraction of that found in the brain (Carraway, 1990; Carraway, 1993; Shaw, 1990). A similar tissue-dependent disparity in precursor processing has been demonstrated in relation to the maturation of other neuropeptides. For instance, pro-opiomelanocortin was shown to be differentially processed in brain and anterior lobe of the pituitary (for a review, see Khachaturian, 1985). Similarily, pro-enkephalin A was found to undergo distinct maturation steps in brain and adrenal medulla (Bair, 1982; Liston, 1984; Liston, 1983). In both of these cases however, the distinct post-translational processing gives rise to different varieties of biologically active compounds. It is not yet clear whether the processing of the Lys^{85}-Arg^{86} doublet reported here in the brain similarily yields (a) biologically active fragment(s).

In the globus pallidus, axon terminals immunoreactive for both E6I and K6L displayed a more extensive distribution than those immunostained for NT. Surprisingly, this relative increase in E6I and K6L immunoreactivity showed up more markedly in immunohistochemical preparations than in radioimmunoassays. Indeed, iE6I/iNT and iK6L/iNT ratios were found by radioimmunoassay to be only 25% and 35% higher, respectively, in the globus pallidus than in whole brain extracts (DeNadai, 1994). This

discrepancy may be explained in part by a dilution of the biochemical values due to the inclusion of [illegible]pallidal areas in the tissue samples. Nonetheless, the immunohistochemical results strongly suggest the existence of a regionally-differential processing of the precursor molecule in certain restricted areas. This interpretation is further supported by the detection of E6I and K6L, but not NT, immunoreactivity in granule cells of the dentate gyrus in colchicine-treated animals. The absence of NT in the dentate gyrus could therefore result from differential release of NT which would govern the relative concentrations of NT susceptible to be detected by immunohistochemistry. Other neuropeptide precursors, including pro-opiomelanocortin (Khachaturian, 1985), pro-enkephalin (White, 1986), pro-dynorphin (Fallon, 1990), and pro-cholecystokinin (Hokfelt, 1985) have been reported to exhibit regionally differential distributions of immunoreactive maturation products in rat brain. The present results suggest that the Lys^{85}-Arg^{86} and Lys^{140}-Arg^{141} dibasics of the NT precursor are cleaved more extensively in certain brain areas, and therefore in certain NT neuronal sub-populations, than in others. Whether this differential susceptibility reflects differential expression and/or activity of processing enzymes remains to be established.

In addition to being more extensive than the NT arbor, E6I- and K6L-immunoreactive axons in the globus pallidus were also as intensely labeled in colchicine-treated as in untreated rats. This was in marked contrast to what was observed in other brain areas where colchicine pretreatment substantially decreased the intensity of terminal immunostaining for all three antigens. A possible explanation for this finding is that the NT innervation of the globus pallidus might originate from neurons in the dorsal medial

part of the neostriatum (Sumigoto, 1987) in which colchicine has been shown to selectively induce increased expression of pro-NT mRNA (Kiyama, 1991). It is intriguing in this regard that these same neurons would be responsible for the differential metabolism and/or release of NN postulated to occur in the globus pallidus on the basis of biochemical experiments (DeNadai, 1994). It might imply that striato-pallidal NT neurons regulate the expression, post-translational processing and post-processing disposal of pre-pro NT and its derivatives in a manner distinct from that of other NT-containing cell groups.

Only a few neuronal cell bodies were detected for all three antigens (i.e., NT, E6I and K6L) in non colchicine-treated rats. This finding suggests that the bulk of NT/NN precursor processing occurs either in, or en route towards, the neuron's terminal arborizations, as previously postulated to be the case for other neurotransmitter precursors including pro-opiomelanocortin (Lis, 1982), the neurophysins (Browstein, 1980) and pro-enkephalin (Liston, 1983). Colchicine pretreatment markedly increased the number and labeling density of NT-immunoreactive cells, but much less so of E6I and particularly of K6L reactive ones. Yet, double labeling studies confirmed that, except in the dentate gyrus, E6I and K6L immunoreactivity was strictly confined to NT-containing cells and was therefore unlikely to be derived from neurons insensitive to the effects of colchicine. The most logical interpretation for the relative paucity of E6I and K6L immunoreactive cell bodies is therefore that the maturation cleavages at the dibasic sites adjacent to these sequences occur further distal to the cell body than those giving rise to NT. Consistent with this possibility is the observation that, in non colchicine-

treated rats, NT immunoreactivity was consistently more intense than that of E6I at the level of nerve cell bodies, only slightly greater than that of E6I in axons (e.g. in the stria terminalis), and virtually identical to that of E6I in terminal fields. The gradient was even more pronounced in the case of iK6L, which was virtually undetectable at the level of neuronal perikarya, but increased progressively in axons and, most notably, in axon terminals. Thus, the present results suggest the existence of an intraneuronal gradient of maturation products, implying that the maturation is not confined to the perikaryal domain but may occur in the course of axonal transport. In precursor that contain multiple cleavage sites, such as the NT/NN precursor, the changes in conformation that accompagny each cleavage may expose new sites to processing enzymes. The NT/NN precursor's maturation might be mediated by sequential removal of the Lys^{163}-Arg^{164} doublet, (thereby exposing the C-terminus of NT), followed by the Lys^{148}-Arg^{149} site (thus liberating NT), and finally the Lys^{140}-Arg^{141} bond (which liberates NN and effectively exposes the E6I sequence). The dynamic control of processing by conformational changes provide an explanation for the sequencial nature of processing mechanism in which cleavage appear to occur in a predetermined order (Smith, 1988). A similar temporal sequence of maturation already postulated to occur in rMTC 6-23 cells (Kitabgi, 1992).

In sum, the present immunohistochemical data confirm and expand upon the biochemical results obtained from experiments carried out in parallel in the laboratory of Dr. Kitabgi (Nice) (DeNadai, 1994). They support the conclusion that in rat brain the three most C-terminal dibasic processing sites in the neurotensin/neuromedin N precursor

are cleaved to a fairly large extent, whereas the dibasic site that precedes the neuromedin N-like sequence is processed to a lesser extent. They also suggest the existence of a regionally differential processing of the NT/NN precursor, particularly with respect to cleavage of the Lys^{85}-Arg^{86} and Lys^{140}-Arg^{141} dibasic doublets. Finally, they suggest the existence of a sequential cleavage of the different dibasic sites in the course of axonal transport from cell body to axon terminals. As discussed in the following section, the double labeling experiments did not only confirm the cellular colocalization of the various segments of the NT/NN precursor, they also provided important information regarding the subcellular distribution of these maturation derivatives and the intracellular location of the post-translational events underlying proNT/NN maturation.

Subcellular distribution of the NT/NN derivatives

To determine the subcellular distribution of the NT/NN precursor maturation products, we carried out an analysis of double-labeled immunocytochemical preparations using the confocal scanning laser microscope (CSLM). Because the spectrograph in the CSLM actually performs as a variable bandpass filter, double-fluorescent images could easily be split into their constituent colors and processed independently. Furthermore, the system allowed for the combination of these individual images, revealing the spatial relationship of the different markers in the specimen. The subcellular distribution of NT has previously been documented by EM in the ventral tegmental area, amygdala and

peripheral nervous system (Bayer, 1991a; Bayer, 1991b; Morales, 1993; Woulfe, 1992), but no data are currently available on the distribution of NT, NN or other NT/NN precursor derivatives throughout the CNS.

The analysis was carried out on both normal and colchicine pre-treated animals to increase the number of immunoreactive perikarya and to control for the possible effects of colchicine on the packaging of newly synthesized molecules. Colchicine has been used to increase perikaryal neuropeptide levels and thereby facilitate the detection of peptidergic nerves cell bodies in the CNS (Butcher, 1983). This increase is usually believed to result from the interruption of axonal flow via the disruption of microtubules. However, a recent report by Kiyama and Emson has shown that colchicine also increased proNT/NN mRNA in particular regions of the brain (Kiyama, 1991), suggesting that the colchicine-induced rise in immunoreactivity might also be due to increased peptide synthesis. In our preparations, no significant difference was observed in the subcellular distribution of immunoreactive NT- and/or E6I between colchicine-treated and control animals in that the forementioned signal was concentrated, in both cases, within intracytoplasmic granules. However, colchicine did increase the intensity of the immunofluorescent signal inside individual granules. Thus, it would appear from our results that the joint effects of colchicine on axonal transport and NT/NN mRNA induction do not affect the packaging of the NT and E6I antigens. Similar conclusions were reached in electron microscopic studies of central noradrenergic neurons which showed that although a massive pile up of noradrenaline storage granules was observed following colchicine pre-treatment, the synthesis and packaging of noradrenaline

proceeded normally (Hokfelt, 1971).

Reconstructed images of individual neurons were generated from the combination of multiple optical sections serially acquired throughout the thickness of double-labeled material. On these reconstructed images, we observed that the NT and E6I immunofluorescent signal was distributed throughout nerve cell bodies, dendrites and terminals, whereas K6L immunoreactivity was predominantly observed in axon terminals. The exact same distribution of immunoreactive signal was observed in our single labeling experiments from which we concluded that a sequential cleavage of the NT/NN precursor takes place in the course of axonal transport from cell body to axon terminals.

Through the means of electronic zooming, we further increased the magnification level in order to display with greater details the subcellular organization of the fluorescent signal. In these high resolution images, a granular pattern of NT, E6I and K6L (where detectable) immunofluorescence was distributed throughout the soma and proximal dendrites of the immunolabeled cells. The average diameter of the fluorescent granules varied between 80nm and 200nm, which is in keeping with the dimension of large dense core vesicles (70-130nm), identified by electron microscopy as intracellular peptide stores (Kandel, 1991). Admittedly, fluorescent particules were larger on average than large dense core vesicles observed with the electron microscope. A possible explanation for this discrepancy is the difference in histological processing between the two types of material. Thus, tissue preparation for electron microscopy includes dehydration, embedding in plastic and polymerization at high temperature, resulting in shrinkage of the specimen. In contrast, histological processing for fluorescent

microscopy does not require dehydration or processing at high temperature, thereby decreasing the risk of morphological alterations.

The predominant localization of perikaryal NT to intracytoplasmic granules reported in the present study is in conformity with the results of an earlier electron microscopic study of the subcellular distribution of NT in the parabrachial pigmentous and paranigral subdivisions of the ventral tegmental area in the brain of colchicine rats (Bayer, 1991a). Indeed, this study reported a predominant association of NT-like immunoreactivity with dense core vesicles at the level of both the soma and proximal dendrites. However, this and other EM studies also reported a diffuse intracytoplasmic localization of NT-immunoreactivity within the perikarya of NT-containing neurons. This diffuse immunoreactivity presumably correspond to the diffuse cytoplasmic immunofluorescent signal detected in our preparations. Admittedly, the diffuse NT-immunoreactivity could also reflect a diffusion of the FITC and /or TR molecules in the cytoplasm of the neuron following degradation of FITC- and TR-labeled IgGs or to a loss of the maturation products from large dense core vesicles in the course of immunolabeling (Novikoff, 1972). In fact, the latter interpretation was involved in a recent study of the ultrastructural localization of NT-immunoreactivity in the cat stellate ganglion, where although the reaction product was mainly concentrated in large dense core vesicles, a floccular NT-like immunoreactivity also pervaded the cytoplasm of the varicosities (Morales, 1993). Nonetheless, the possibility remains that this diffuse intracellular signal correspond to a cytoplasmic pool of NT/NN precursor derivatives.

The observation that the E6I and K6L (when detectable) antigens are present in

granular structures suggests that NN and other maturation products of the NT/NN precursor are also packaged into large dense core vesicles. With reference to NN, it is noteworthy that a single $LYS^{(140)}$-$Arg^{(141)}$ dibasic bonds separates the NN sequence and the E6I epitope in the primary structure of the precursor. This fact combined with evidence that the cleavage at this dibasic site both releases NN from the precursor (Bidard, 1991) and exposes the E6I epitope (DeNadai, 1994) implies that the localization of E6I immunoreactivity reflects the regional and cellular domain of final NN maturation. Consequently, the granular E6I immunofluorescent signal observed in neuronal perikarya suggests that NN is processed within secretory vesicles.

Combination of individually processed images for NT and E6I immunolabeling indicated that NT and E6I immunoreactivity were colocalized at the level of individual granules. Since, as described above, the E6I signal may be considered as an indicator of the subcellular localization of NN, our results suggest that NT and NN are colocalized in the same secretory vesicles. They also imply that NN is co-released with NT, an interpretation congruent with the observation that these two peptides are co-released in a calcium-dependent manner from hypothalamic slices in vitro (Kitabgi, 1990).

The predominant association of NT and NN (i.e E6I) immunoreativity with intracytoplasmic granules observed here in neuronal cell bodies of both normal and colchicine treated-animals suggests that cleavage of the NT/NN precursor at the dibasic bounds exposing the E6I site and liberating NT occurs after budding of the secretory granule from the *trans*-Golgi network. Indeed, if this primary cleavage of the precursor had taken place before the formation of the vesicle in the *trans*-Golgi network, one would

have expected a preferential accumulation of immunofluorescent labeling in the perinuclear region, where the Golgi apparatus is normally found. In contrast, the labeling appeared randomly distributed throughout the cytoplasm of the perikarya. Furthermore, given that the subcellular localization of endoproteolytic cleavages is a determining parameter in regulating the processing and packaging of propeptide products (Sossin, 1989), cleavage in the Golgi should have resulted in the segregation of the peptide products into separate vesicles. Instead, we observed a strict colocalization of NT-and E6I-immunoreactivity within the same granule-like structures which supports the interpretation that prohormone processing occurs following the budding of the immature secretory granules from the *trans*-Golgi network. In agreement with our data is the report by Gainer and colleagues (Gainer, 1977), demonstrating that oxytocin and vasopressin are being released from their respective prohormone while being transported down the axon. Furthermore, the use of an antibody specific for an endoproteolytic site provided evidence that pro-opiomelanocortin is also packaged into secretory vesicles before its cleavage (Tooze, 1987). Although our data suggest that processing of NT/NN precursor predominantly occurs outside the Golgi, they do not entirely exclude the possibility that part of proteolytic cleavage occurs in the *trans*-Golgi network prior to packaging of intermediate products in the same vesicle. A final setting of this issue obviously requires identification of the various cellular subcompartments using specific markers such as acid phosphatase for the *trans*-Golgi network.

The fact that NT and NN are derived from a large molecular weight precursor implies the existence of an enzyme capable of cleaving preferentially at recognized pairs

of basic amino acids. Yet, no information is available concerning the converting enzymes responsible for the processing of the NT/NN precursor. Despite the recent identification of several key endoproteases, little is known about their proprotein substrate(s). The emerging characteristics of two of these convertases, PC1 and PC2, which are both present in high concentrations in the brain suggested to us that they might represent endoproteases involved in the proteolytic processing of the NT/NN precursor.

NT and the processing enzymes PC1 and PC2

Many characteristics exhibited by the PC1 and PC2 members of the subtilisin family indicate that these two enzymes could represent the neural endoproteases involved in propeptide processing (Benjannet, 1991; review). Indeed, both PC1 and PC2 demonstrate tissue specific expression, selectivity of cleavage at pairs of basic residues and substrate specificity (Benjannet, 1991; Rangaraju, 1991; Seidah, 1992). The detection of PC2 and PC1 exclusively in cells of neuroendocrine origin (Seidah, 1991; Smeekens, 1992) together with their restricted and sometimes overlapping regional distribution of these two enzymes in peptide-rich regions of the brain (Cullinan, 1991) suggested that a particular combination of these enzymes might be responsible for the regionally specific processing of neuropeptide precursors, including the NT/NN precursor. Hence, comparing the tissue distribution of PC1 and PC2 in relation to the previously characterized distribution of NT can provide insight into the possible role of these enzymes in the maturation of the NT/NN precursor.

Our comparative analysis was not meant to provide an exhaustive description of the distribution of PC1 and PC2 immunoreactivity throughout the rat brain. Instead, we strictly focussed on NT-rich regions in order to determine whether or not these enzymes were codistributed with the NT/NN precursor. Among the regions selected for examination were the lateral septal nucleus, the preoptic area, the nucleus accumbens, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the paraventricular nucleus of the thalamus and the hypothalamus, all regions found in a number of studies to contain high concentrations of NT-immunoreactive cell bodies and/or axon terminals.

In all regions examined, the topographical distribution of PC1-immunoreactive cells was highly restricted and correlated only marginally with the extensive distribution of NT-immunoreactive neurons. Thus, low to undetectable levels of PC1-immunoreactive signal were detected in the caudo-putamen, the preoptic area, the bed nucleus of the stria terminalis and the arcuate nucleus, all regions found in adjacent sections to contain a large number of NT-immunoreactive cells. Moderately-labeled PC1 neurons were prevalent in the paraventricular nucleus of the thalamus as well as in the lateral hypothalamus but although their distribution was similar to that of NT in the former, it was strikingly different in the latter. Finally, no PC1-immunoreactive perikarya were detected in the lateral septum, the nucleus accumbens nor the central nucleus of the amygdala where a large number of NT-immunoreactive cells was also detected.

Although the distribution of PC1-immunoreactive neurons generally conformed

with the distribution neurons previously shown by *in situ* hybridization to contain PC1 mRNA (Schafer, 1993), the intensity of PC1-immunoreactive signal didn't always correlate with the levels PC1 mRNA expression within the same regions. For instance, whereas the absence of PC1-immunoreactive signal in the nucleus accumbens, the central nucleus of the amygdala and the lateral septum nucleus conformed with *in situ* hybridization studies reporting either none or close to undetectable levels of PC1 mRNA expression in these nuclei, the fact that only low levels of PC1 immunoreactive signal were detected within the preoptic area, bed nucleus of the stria terminalis, arcuate and paraventricular nucleus of the hypothalamus is at odds with previous *in situ* hybridization findings of moderate to high levels of PC1 mRNA in these regions (Cullinan, 1991; Schafer, 1993). Similarly, high PC1-mRNA expression levels were detected in the paraventricular nucleus of the thalamus and the lateral hypothalamus while only a moderate to low PC1-immunoreactive signal was detected by immunocytochemistry. The most likely explanation for these discrepancies is the fact that our PC1 antiserum was directed against the c-terminal portion (98 aa) of the protein (Benjannet, 1992), a segment likely to be excised in the course of post-translational modifications (Benjannet, 1993; Vindrola, 1992). Indeed, using antisera directed against the amino-terminal portion of the mature PC1 protein, pulse-chase/immunoprecipitation experiments in AtT-20 cells demonstrated that the original 87KDa PC1 protein was converted into a 66KDa product in a time-dependent fashion (Vindrola, 1992). Hence, the PC1-immunoreactive signal detected in our preparations likely corresponds to the fraction of PC1 enzymes recently translated from PC1 mRNA but not yet cleaved into the lower molecular form. The

discrepancy noted here between the levels of PC1 mRNA expression and the intensity PC1-immunoreactive signal might also result from a differential translation of PC1 mRNA into the PC1 protein in different neuronal populations. Indeed, regions reported to express identical levels of PC1 mRNA expression, eg. the paraventricular nucleus of the thalamus and the arcuate nucleus of the hypothalamus, exhibited different intensities of PC1 immunoreactive signal. But then again, given that our anti-PC1 antisera detects only the large (84Kd) form of PC1, the regional variation in the intensity of PC1-immunoreactive signal could reflect a difference in the extent to which the 84KDa protein is truncated to its lower molecular form. This appears all the more likely since the extent to which this cleavage occurs was shown to be cell-type dependent (Benjannet, 1993; Vindrola, 1992).

The absence of PC1-immunoreactive signal in regions such as the central nucleus of the amygdala, the nucleus accumbens and the lateral septum, where NT-immunoreactive cell bodies are prevalent, strongly suggests that (an) other enzyme(s) is/are involved in the proteolytic cleavage of the NT/NN precursor. This interpretation is further supported by the difference in distribution between NT- and PC1-immunoreactive neurons in the preoptic area and lateral hypothalamus and the fact that in other regions, such as in the bed nucleus of the stria terminalis, the arcuate nucleus and the paraventricular nucleus of the hypothalamus, NT-immunoreactive cells far outnumbered the PC1-immunoreactive neurons. However, in these later areas, and particularly in the paraventricular nucleus of the thalamus where we noted a similitude in the density and distribution of PC1 and NT-immunoreactive neurons, there could exist

a partial co-localization of PC1 and NT. This possibility is made further likely by the fact that our antibody recognizes only the c-terminus of the enzyme and that the number of PC1-containing neurons is probably undeterestimated. To settle this issue it will be necessary to conduct a detailed comparative analysis using a N-terminally directed PC1 antiserum. Only with this extensive mapping will it be possible to further characterize the overlap of PC1 with NT and infer about the potential role of PC1 in the proteolytic processing of the NT/NN precursor.

The immunocytochemical distribution of PC2 in our preparations generally correlated much more closely with the PC2 mRNA expression patterns obtained by in situ hybridization than that of PC1 (Schafer, 1993). Thus, moderate to high intensities of PC2 immunolabeling were prevalent in those regions where moderate to intense PC2 mRNA levels were detected. Furthermore, no PC2 immunoreactive signal was detected in the central nucleus of the amygdala, a region clearly devoided of PC2 mRNA. However, the PC2-immunoreactive signal was weak in the nucleus accumbens and the arcuate nucleus, two nuclei shown by *in situ* hybridization to exhibite moderate levels of PC2 mRNA. The possibility that our anti-PC2 antibody recognizes only a fraction of the enzyme cannot be invoked to explain the relatively low PC2 signal observed in the nucleus accumbens and in the arcuate nucleus since even though our the anti-PC2 antiserum is directed against the c-terminal segment of the protein, there is no evidence for a C-terminally trucated form of PC2 (Kirchmair, 1992a; Vindrola, 1992). Nevertheless, as suggested for PC1, the discrepancy observed between the levels of PC2 mRNA and that of PC2-immunoreactive signal could result from differential translation

of the PC2 mRNA.

The topographical overlap between PC2 and NT immunolabeling was considerably more extensive than what observed in the case of PC1. Thus, coexistence of NT and PC2 positive neurons was observed in the bed nucleus of the stria terminalis, the lateral septal nucleus, the paraventricular nuclei of the thalamus and the hypothalamus, the preoptic area and the lateral hypothalamus. However, when the comparative analysis was carried out at the sub-regional level, the distribution and/or cytological characteristics of NT- and PC2-reactive neurons were often different. Thus, in the bed nucleus of the stria terminalis, the highest density of NT positive cells was observed in the lateral subdivision, whereas PC2-immunoreactive cells were predominantly found in the medial aspect. Similarly, in the lateral septum, NT-immunoreactive cells were present throughout the nucleus whereas the PC2-positive neurons were predominantly localized in the dorsal subdivision. In the preoptic area, NT-labeled neurons were scattered throughout the structure, whereas the PC2-labeled ones were prevalent mainly in the lateral aspect. Finally, in the medial aspect of the caudo-putamen, immunolabeled NT cells formed a rim along the lateral ventricle, whereas the PC2-reactive neurons were dispersed throughout the entire structure. Thus, although codistributed at the topographical level, NT and PC2 are not necessarily colocalized at the cellular level. Nevertheless, there is a clear possibility that sub-populations of cells in these regions, namely in the dorsal subdivision of the lateral septal nucleus and in the bed nucleus of the stria terminalis, do contain both NT and PC2 and therefore that PC2 might be involved in the proteolytic processing of the NT/NN precursor. This possibility appears

particularly likely in the case of the paraventricular nucleus where the size and distribution of NT- and PC2-immunoreactive neurons was strikingly similar. However, it is well documented that the presence of the NT/NN precursor in this region is induced by colchicine pretreatment (Kiyama, 1991), implying that cleavage of the NT/NN precursor by PC2 in the paraventricular nucleus would result strictly from the colchicine-induced expression of the NT/NN precursor.

The absence of PC2 immunoreactive signal in NT-rich regions such as the central nucleus of the amygdala, the nucleus accumbens and the arcuate nucleus implies that at least another enzyme in involved in the proteolytic cleavage of the NT/NN precursor. It is interesting in this context that PC5 was found to be highly concentrated in the central nucleus of the amygdala (Lusson, 1993) which makes it a putative candidate for NT/NN precursor processing in this region.

Based on our observations, it seems probable that other kinds of proteases, structurally related or not with the subtilisin family, may be involved in the processing of the NT/NN precursor. In fact, the relative contribution of various enzymes toward the maturation of various precursor has been postulated to vary according to brain regions (Bloomquist, 1991; Schafer, 1993) In turn, the nature of the enzymatic combination might lead to the regional differences in the processing of the NT/NN precursor observed in the first part of this work.

In sum, the regional co-distribution in some regions of NT with PC1 and/or PC2 suggests a possible involvement of PC2, but less likely of PC1, in the proteolytic processing of the NT/NN precursor. However, differences in the cytological features of

PC1-, PC2- and NT- containing neurons together with the absence of either PC1 or PC2 immunoreactivity in NT-rich regions strongly suggest that PC1 and PC2 are not the only endoproteases involved in the maturation of the NT/NN precursor.

CONCLUSION

The immunocytochemical identification of the NT/NN precursor derivatives in the rat CNS suggested that the three most C-terminal dibasic sites in the precursor's primary structure are cleaved to a fairly large extent whereas the dibasic site that precedes the NN-like structure (K6L) is processed to a lesser extent. Mapping of the neuronal populations expressing those derivatives suggested the existence of a regionally differential processing of the precursor, particularly with respect to cleavage of the Lys^{85}-Arg^{140} dibasic doublets, which appear to be more extensive in some regions than in others. Furthermore, the comparison of the relative densities of immunolabeling for the maturation products at the level of cell bodies and nerve terminals, suggested the existence of a sequential cleavage of the different dibasic sites in the course of axonal transport from the cell body to the nerve terminals. The double labeling experiments and the analysis by confocal microscopy confirmed the cellular colocalization of the various maturation derivatives. Moreover, it provided important informations regarding the subcellular distribution of these maturation derivatives as well as the intracellular events underlying proNT/NN maturation. Thus, the predominant association of NT and NN (i.e.E6I) immunoreactivity with cytoplasmic granules suggests that cleavage of the NT/NN precursor at the dibasic bonds exposing the E6I sequence and liberating NT occurs after budding of the secretory granules from the *trans* golgi network.

The fact that NT and NN are derived from a large molecular weight precursor implies the existence of (an) enzyme(s) capable of cleaving preferentially at recognized

pairs of basic amino acids. The comparative analysis of the tissue distribution of the PC1 and PC2 maturation enzymes in relation to the previously characterized distribution of NT provided some insights into the possible role of these enzymes in the maturation of the NT/NN precursor. On one hand, the regional co-distribution in some regions of NT with PC1 and/or PC2 suggests a possible involvement of PC2, but less likely of PC1, in the proteolytic processing of the NT/NN precursor. On the other hand, the differences in the cytological features of PC1-, PC2- and NT-containing neurons together with the absence of either PC1 or PC2 immunoreactivity in NT-rich regions strongly suggest that PC1 and PC2 are not the only endoproteases involved in the maturation of the NT/NN precursor.

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