Immunohistochemical and Biochemical Evidence for a Role of Prohormone Convertases PC1, PC2 and PC5A in the Endoproteolytic Processing of the Neurotensin/Neuromedin N Precursor in Rodent Brain

by

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To my parents,
To my brothers and sister

THE POWER OF THOUGHT

"As you think, you travel; and as you love, you attract. You are today where your thoughts take you. You cannot escape the results of your thoughts, but you can endure and learn; can accept and be glad.

"You will realize the vision, not the idle wish, of your heart, be it base or beautiful, or a mixture of both, for you will always gravitate toward that which you secretly most love.

"Into your hands will be placed the exact results of your thoughts; you will receive that which your earn-no more, no less.

"Whatever your present environment may be, you will fall, remain, or rise with your thoughts, your vision, your ideal.

"You will become as small as your controlling desire; as great as your dominant aspiration".

Author unknown

LIST OF ABBREVIATIONS

NT:	Neurotensin
NN:	Neuromedin N
Pro-NT/NN:	Pro-neurotensin/neuromedin N
E6I:	Abbreviation for pro-NT/NN peptide fragment containing the amino acids Glu-Lys-Val-Glu-Glu-Ile
K6L:	Abbreviation for pro-NT/NN peptide fragment containing the amino acids KLPLVL
iNT, iNN:	Immunoreactive NT and NN, respectively
HPLC:	High pressure liquid chromatography
RIA:	Radioimmunoassay
CTiNN:	Citraconylated, trypsin-digested iNN
PC:	Prohormone convertase
CPE:	Carboxypeptidase E
ACTH:	Adrenocorticotrophin hormone
POMC:	Pro-opiomelanocortin
RE:	Endoplasmic reticulum
TGN:	*Trans*-Golgi network
CNS:	Central nervous system
WT:	Wild-type
ip:	Intraperitoneal
icv:	Intracerebroventricular
CSF:	Cerebrospinal fluid

ABSTRACT

Proteolytic maturation of neuropeptide precursors is a general mechanism through which a variety of neuropeptides are synthesized in the mammalian brain. Cumulating evidence suggests that this process is mediated by enzymes of the prohormone convertase (PC) family. However, the precise role and mode of action of these PCs in the brain are still poorly understood. To gain further insight in the putative function of PC5A in the processing of neuropeptide precursors in the brain, we examined its regional, cellular and subcellular distribution in rat brain using immunohistochemistry at the light and confocal microscopic levels. PC5A was extensively distributed throughout the rat brain. It was present in selective neuronal population, but not in glia, consistent with a role of this enzyme in the maturation of peptide precursors destined for regulated secretion. At the subcellular level, PC5A was mainly concentrated in the Golgi apparatus and perikaryal vesicles, suggesting an implication of the enzyme early in the neurosecretory pathway. In a second set of studies, the immunohistochemical distribution of neurotensin (NT) and of either PC1, PC2 or PC5A were compared in rat brain sections using single- or double-labelling immunohistochemistry in order to ascertain whether these convertases might play a role in the processing of the NT precursor (pro-NT/NN) *in vivo*. The proportion of NT immunoreactive neurons co-expressing either one of these PCs was quantified in NT-rich regions. Neurons co-localizing NT and PC2 were the most numerous, with over 60% of NT-immunoreactive cells being also positive for PC2. NT/PC1 and NT/PC5A neurons were less numerous. Furthermore, the distribution of NT neurons co-localizing PC1 was negatively correlated with that of NT neurons expressing PC5A, suggesting that PC1 and PC5A may play interchangeable roles for the processing of pro-NT/NN in

mammalian brain. Using confocal microscopic immunohistochemistry, the bulk of PC1 and PC5A were found to co-localize with the Golgi protein MG-160, whereas PC2 was mostly excluded from MG-160-containing compartments. This suggests that PC1 and PC5A might be involved in the early steps of pro-NT/NN processing, whereas PC2 would act later along the secretory pathway. To gain further insight into the possible involvement of these PCs in the *in vivo* processing of pro-NT/NN, the maturation profile of pro-NT/NN was investigated by radioimmunoassay using site-directed antibodies in the brain of mice that were inactivated for PC2. There was a markedly impaired processing of pro-NT/NN in PC2 null mice compared to wild type. The defects were very specific and resulted in a 15% decrease in mature NT and a 50% decrease in NN as compared to wild-type mice. Furthermore, we showed by immunohistochemistry that the defects preferentially affected specific regions of the brain, namely the medial preoptic area, lateral hypothalamus, arcuate and paraventricular hypothalamic nuclei. These findings indicate that PC2 plays a critical and specific role for the physiological processing of pro-NT/NN in the rodent brain and confirm that other PCs, namely PC1 and/or PC5A, are also involved.

RÉSUMÉ

La maturation protéolytique de précurseurs peptidiques est un mécanisme général de biosynthèse des peptides dans le cerveau des mammifères. Ce processus s'exerce par l'intermédiaire d'enzymes de la famille des prohormones convertases (PCs). Cependant, le rôle précis ainsi que le mode d'action des PCs dans le cerveau demeurent mal définis. Afin de mieux comprendre le rôle de la prohormone convertase PC5A dans la maturation des neuropeptides cérébraux, la distribution régionale, cellulaire et subcellulaire de cette enzyme a été examinée dans le cerveau du rat par des techniques d'immunohistochimie en microscopie photonique et confocale. La PC5A est abondamment distribuée dans le cerveau de rat. Son expression exclusivement neuronale est en accordance avec son implication dans la maturation de nombreux précurseurs peptidiques sécrétés dans la voie régulée de sécrétion. Au niveau subcellulaire, la PC5A est principalement concentrée dans l'appareil de golgi, mais également au niveau de vésicules intra-périkaryales, suggèrant qu'elle joue un rôle précoce dans la voie régulée de la sécrétion. Afin de déterminer si les prohormones convertases PC1, PC2 et PC5A pourraient être impliquées dans la maturation du précurseur de la neurotensine (NT; pro-neurotensin/neuromedin N) au niveau du système nerveux central, la distribution de la NT a été comparée à celle de la PC1, de la PC2 et de la PC5A à l'aide de techniques de simple et de double marquage immunohistochimique en microscopie photonique. Nous avons également quantifié les proportions relatives de neurones neurotensinergiques co-exprimant soit PC1, PC2 ou PC5A dans les régions où la NT est fortement exprimée. Des trois PCs, PC2 est la plus souvent co-exprimée avec la NT puisqu'en moyenne, plus de 60% des neurones neurotensinergiques en possèdent. La NT est moins largement co-exprimée avec la PC1 et la PC5A. De plus, on note une corrélation inverse entre les neurones exprimant NT/PC1 et ceux exprimant NT/PC5A, ce qui suggère que ces deux PCs sont interchangeables dans la maturation de la pro-NT/NN. Au niveau subcellulaire, la PC1 et la PC5A, mais non la PC2, sont fortement co-localisées avec le marqueur golgien MG-

160. Ces données indiquent que la PC1 et la PC5A sont toutes deux probablement impliquées dans les étapes précoces de la maturation endoprotéolytique de la pro-NT/NN, alors que la PC2 est impliquée plus tardivement. Finalement, afin de mieux cerner le rôle de la PC2 dans la maturation endoprotéolytique de la pro-NT/NN *in vivo*, la maturation de ce propeptide a été examinée par immunohistochimie et radioimmunologie chez des souris dont le gêne codant pour la PC2 a été invalidé. Le dosage radioimmunologique des produits de maturation de la pro-NT/NN montre une diminution de 15% de la NT mature et une diminution de 50% de la production de la NN chez les souris invalidées par rapport aux souris contrôles. De plus, l'analyse immunohistochimique du cerveau de ces animaux montre que ces déficits sont particulièrement marqués dans certaines régions en particulier. En effet, une diminution marquée de l'immunoréactivité des produits de maturation de la pro-NT/NN est détectée dans l'aire préoptique médiane, l'hypothalamus latéral et le noyau arqué de l'hypothalamus, mais pas dans les autres régions cérébrales examinées. En conclusion, ces résultats démontrent que la PC2 joue un rôle pivot dans la maturation endoprotéolytique de la pro-NT/NN dans le système nerveux central et confirment que d'autres PCs, dont PC1 et PC5A, y jouent aussi un rôle.

CLAIM FOR ORIGINAL WORK

The results presented in this thesis represent an original contribution to knowledge on the roles of prohormone convertases in peptide synthesis of the neuropeptide neurotensin in the mammalian brain. Much of this work has been presented both orally and in poster form at various scientific meetings and symposia including the 2000 Summer Neuropeptide Conference and Gordon Conference on Hormonal and Neural Peptide Biosynthesis, the 2001 Peptide and Peptide Receptors meeting, the Annual Clinical and Investigative Medicine meeting of the Royal College of Physicians of Canada and the 1997 and 2001 Annual Meetings of The Society for Neuroscience.

Chapter 1 presents a review of the research literature pertaining to neurotensin, with an emphasis on how the regulation of its biosynthesis, release, action on target neurons and degradation may influence its biological activity. A review of the literature on prohormone convertases is also presented, particularly concerning their role in neuropeptide synthesis and possible implication in pathological conditions.

In Chapter 2, a thorough description of the immunohistochemical distribution of the prohormone convertase PC5A in the adult rat brain is presented. PC5 was found to be expressed selectively by neurons, supporting the view that it plays a role in the processing of precursors routed in the regulated secretory pathway. Its widespread distribution suggests that it is involved in the processing of numerous peptide precursors throughout the rat brain.

The papers presented in Chapters 3 and 4 represent the first effort to systematically compare the distribution of several members of the convertase family with that of one of their potential substrate. Specifically, we used immunohistochemistry to determine whether the prohormone convertases PC1, PC2 and PC5A were co-localized in the same neurons as pro-NT/NN and could thus play a role in the physiological processing of this propeptide. Our results demonstrate partial co-localization of all three convertases with NT throughout the rat brain. Moreover, the proportion of NT neurons co-localizing with all three convertases was highly region-specific, probably accounting for the regional differences in pro-NT/NN processing reported in earlier studies. Our results also strongly suggest that several PCs may be co-expressed within single neurons, which, in addition to provide the system with redundancy, could help fine-tuning the processing events.

In Chapter 5, the physiological role of PC2, PC1 and PC7 in pro-NT/NN processing were evaluated by comparing the processing profile of pro-NT/NN in wild type vs mice inactivated for PC7 (PC7-/-), PC2 (PC2-/-) and/or PC1 (PC1+/- and PC2-/-; PC1+/-). Our data suggest that PC7 does not play a role in the processing of the NT/NN precursor. However, they strongly support the contention that PC2 plays a pivotal role in pro-NT/NN processing, in particular for the release of NN from the precursor. Moreover, we present the first evidence demonstrating that the effects of PC2 invalidation affected the processing of a peptide precursor, namely pro-NT/NN, in a region-specific manner. PC1+/- mice did not differ from WT in their processing of pro-NT/NN, implying that complete invalidation of PC1 may be required for lost of function.

Chapter 6 presents a general discussion which interrelates the results of the previous chapters and evaluates their overall significance from a functional perspective.

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Publications

Articles:

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Contributions of authors

The following is a description of the contribution by each co-author to the conception, experimental design, analysis and writing of the material presented in the four manuscripts. With the exception of the contributions listed below, I claim responsibility for the experimental design, experimental manipulation and data analysis relevant to each study as well as the preparation of the manuscript themselves. In addition, Dr. Alain Beaudet contributed substantially, in the capacity of supervisor and co-author, to the work presented the manuscripts. He offered sound advice and counselling from both a technical and conceptual perspective throughout the duration of the study. Moreover, his contribution to the preparation of each manuscript in the form of constructive criticism as well as meticulous editing and revision was invaluable.

Manuscript 1: *Immunohistochemical Distribution of the Prohormone Convertase PC5A in Rat Brain.*

Authors: Pierre Villeneuve, Nabil G. Seidah and Alain Beaudet.

Contributions: Dr Seidah supplied us with the PC5A antibody and cognate peptide necessary to perform the study. He also contributed to the final editing of the manuscript. Mariette Houle, our technician, prepared the photographic plates.

Manuscript 2: *Immunohistochemical Evidence for the Involvement of the Protein Convertases PC5A and PC2 in the Processing of Pro-Neurotensin in Rat Brain.*

Authors: Pierre Villeneuve, Louise Lafortune, Nabil G. Seidah, Patrick Kitabgi and Alain Beaudet.

Contributions: Louise Lafortune, a former Master student in our laboratory, performed pilot PC2 immunohistochemical studies and carried out NT/E6I double-labelling experiments. Dr Seidah supplied us with the PC5A antibody and cognate peptide necessary to perform the study and he also contributed to the final editing of the manuscript. Patrick Kitabgi provided us with the E6I antibody and was involved both in the design, analysis and write up of the study. Carole Rovère, a researcher in the laboratory of Dr Kitabgi, performed the immunoblot experiments presented in this study. Mariette Houle contributed to the preparation of the photographic plates.

Manuscript 3: *Immunohistochemical Evidence for the Implication of PC1 in the Processing of Pro-neurotensin in Rat Brain.*

Authors: Pierre Villeneuve, Nabil G. Seidah, and Alain Beaudet.

Contributions: Dr Seidah supplied us with the PC5A antibody and cognate peptide necessary to perform the study and he also contributed to the final editing of the manuscript. Mariette Houle contributed to the preparation of the photographic plates.

Manuscript 4: *Altered processing of the neurotensin/neuromedin N precursor in mice inactivated for PC2: A biochemical and immunohistochemical study.*

Authors: Pierre Villeneuve, Sylvain Feliciangeli, Gilles Croissandeau, Majambu Mbikay, Nabil G. Seidah, Patrick Kitabgi and Alain Beaudet.

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TABLE OF CONTENT

LIST OF FIGURES AND TABLES

Chapter One

Introduction

The importance of peptides as chemical messengers is now well established. In the periphery, they act as hormones critical for the maintenance of homeostasis; in the central nervous system, they exert functions as neurotransmitters/neuromodulators in addition to being critical in the regulation of the neuroendocrine system. It has been recognized for some time that peptides are derived from large polypeptide precursors which are biologically inactive (Chretien and Li, 1967; Steiner, 1967; Loh et al., 1984). These precursors contain one or several copies of different biologically active peptides that are released by proteolytic processing (Loh et al., 1984). The mechanisms underlying endoproteolytic maturation of propeptides have long remained elusive. The aetiology of various endocrine disorders, including glycoregulation disorders, insulin resistance, congenital ACTH deficiency, and hypoparathyoidism have recently been associated with disorders of endoproteolytic processing (Raffin-Sanson and Bertagna, 1997). The recent cloning of enzymes involved in the proteolytic processing of propeptides has made possible the *in vitro* demonstration of this process and shed some light on the molecular machinery involved. Using recombinant cell expression systems, these enzymes were shown to possess the ability to process a variety of propeptides, as well as a number of other precursor molecules, including precursors of enzymes, growth factors, coagulation factors and viral proteins. However, the *in vivo* properties of these proteases still need to be characterized. The demonstration that the common precursor of the biologically active peptide neurotensin (NT) and neuromedin N (NN), pro-NT/NN, undergoes tissue-specific maturation (Carraway and Mitra, 1987, 1990) and the characterization of endoproteases capable of processing the pro-NT/NN precursor *in vitro* (Rovere et al., 1996a; Barbero et al., 1998), have prompted us to attempt to investigate the mechanisms

that may be involved in the processing of this precursor protein in the mammalian brain *in vivo*.

1.1 NEUROTENSIN

1.1.1 Discovery and evidence for a neurotransmitter role

NT was discovered by Carraway and Leeman (1973). While attempting to purify substance P from bovine hypothalamic extracts, they isolated a compound eluting before substance P and displaying upon intravenous injection potent vasodilatation in exposed cutaneous regions of the rat. This compound differed from substance P in its amino acid sequence (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH; Carraway and Leeman, 1975) and was given the name "neurotensin" (NT) after the tissue from which it was purified and because of its hypotensive property (Carraway and Leeman, 1973). NT was later isolated from various tissues from a number of mammalian species (Kitabgi et al., 1976; Uhl and Snyder, 1976; Kobayashi et al., 1977; Kataoka et al., 1979; Hammer et al., 1980; Manberg et al., 1982; Papadopoulos et al., 1986) as well as from birds, amphibians, reptiles, invertebrates and even from primitive species such as protozoans and bacteria (Langer et al., 1979; Carraway and Bhatnagar, 1980; Reinecke et al., 1980; Bhatnagar and Carraway, 1981; Grimmelikhuijzen et al., 1981; Goedert et al., 1984).

NT was shown to be expressed throughout the central and peripheral nervous system where cumulating evidence has suggested that it acts as a *bona fide* neurotransmitter. Thus, immunohistochemical and *in situ* hybridisation studies have shown that NT is selectively expressed by neurons, heterogeneously distributed throughout the neuraxis (Jennes et al., 1982; Alexander et al., 1989; Woulfe et al., 1994); NT has been observed

in nerve terminal vesicles by electron microscopy (Uhl and Snyder, 1976); it is released in a Ca^{2+}-dependent, K^{+}-evoked fashion from live hypothalamic slices (Iversen et al., 1978); NT elicits the activation of target neurons by binding to specific, biologically active receptors (see section 1.1.8). The distribution of at least one of the NT receptor subtype is compatible with its purported interaction with NT in the brain (Kitabgi et al., 1977; Quirion et al., 1982; Tanaka et al., 1990; Nicot et al., 1994; Vincent, 1995; Boudin et al., 1996); central injection of neurotensin produces a number of effects, modulating a variety of autonomic, sensorimotor, and cognitive functions including regulation of body temperature, food intake, sensitivity to alcohol, regulation of mesocortical, mesostriatal and mesolimbic dopamine systems, antinociception and regulation of the sleep-wake cycle (for review see Kitabgi and Nemeroff, 1992). NT has also been implicated in the central regulation of the release of various pituitary hormones, including adrenocorticotrophin hormone, luteotrophic hormone, thyroid stimulating hormone, and growth hormone (Rostene and Alexander, 1997). These central effects of NT will be discussed in more detail later. Finally, the effects of NT are rapidly terminated following synaptic release due to degradation of the peptide into inactive fragments by specific endopeptidases (Dupont and Merand, 1978; Checler et al., 1983; Kitabgi et al., 1992b).

NT is also highly expressed in the gut (90% of total body content) where it is present in endocrine N cells of the small intestine. In the gut, NT is released following meal intake and, through endocrine and paracrine fashion, it stimulates gastric and pancreatic secretion and decreases contraction of smooth muscles lining the gastro-intestinal tract (Vincent, 1995). NT is also detected in the adrenal medulla and thymus, where its functions are still unknown (for review see; Aronin et al., 1983; Carraway and Reinecke,

1984; Kitabgi et al., 1989; Kasckow and Nemeroff, 1991; Rostene and Alexander, 1997; Tyler-McMahon et al., 2000).

1.1.2. Central role of neurotensin

Shortly after the discovery of NT, it was rapidly realized that its intracerebral or intraventricular injection elicits behavioural and physiological effects similar to those induced by neuroleptic drugs. This led Nemeroff to hypothesize that NT might be an endogenous neuroleptic (Nemeroff, 1980). A sum of behavioral, electrophysiological, anatomical and biochemical evidence has since suggested that NT indeed shares some effects in common with neuroleptics:

-Hypothermia (Bissette et al., 1976; Nemeroff et al., 1979).

-Potentiation of barbiturate- and ethanol-induced sedation (Nemeroff et al., 1977; Bissette et al., 1978).

-Motor hypoactivity (Jolicoeur et al., 1981; van Wimersma Greidanus et al., 1982; van Wimersma Greidanus et al., 1984).

-Decreased muscle tone (Osbahr et al., 1979; Snijders et al., 1982).

-Inhibition of avoidance but not escape responding, in a conditioned avoidance paradigm (Osbahr et al., 1979; Luttinger et al., 1982a; Snijders et al., 1982).

Substantial evidence suggests that the neuroleptic-like effects of NT are mediated via an interaction between NT and dopamine (Osbahr et al., 1979; Nemeroff, 1980). Anatomically, interactions between NT and mesostriatal (substantia nigra to neostriatum), mesocortical (ventral tegmental area to prefrontal cortex) and mesolimbic (ventral tegmental area to nucleus accumbens) dopamine pathways have been heavily

documented ; for review see Kasckow and Nemeroff, 1991; Beaudet and Woulfe, 1992). Injection of NT and other NT receptor agonists produces an enhanced dopamine turnover, as evidenced by increases in its metabolites dihydroxyphenylacetic acid (DOPAV) and homovanillic acid (HVA) in various brain areas, including the nucleus accumbens, neostriatum, hypothalamus, olfactory tubercle, septum and frontal cortex (Nemeroff et al., 1982; Widerlov et al., 1982a; Nemeroff et al., 1983; Nemeroff, 1986). In addition, chronic and subchronic administration of all clinically effective typical antipsychotics tested to date have specific effects on the NT system, by producing selective increases in NT immunoreactivity and mRNA in the nucleus accumbens and dorsolateral portion of the caudate-putamen (terminal regions of the mesolimbic and nigrostriatal DA neurons, respectively; Govoni et al., 1980; Merchant et al., 1988; Merchant et al., 1991; Merchant et al., 1992; Merchant and Dorsa, 1993; for review see Kinkead et al., 1999). These effects on the NT system are selective for drugs with antipsychotic efficacy; compounds from other classes of clinically used psychoactive drugs (e.g., anxiolytic, antidepressants, and antihistamines) as well as clinically ineffective phenothiazimes, do not affect the NT system (Govoni et al., 1980; Myers et al., 1992).

Interestingly, typical and atypical antipsychotics differentially regulate the NT system in that typical antipsychotics have actions on both the mesolimbic and nigrostriatal NT systems, whereas atypical antipsychotic drugs act preferentially on the mesolimbic NT system (Kilts et al., 1988; Merchant et al., 1994). This last finding has given rise to the hypothesis that the nigrostriatal NT system may play a role in the side-

effect profile of typical antipsychotic drugs, while the mesolimbic NT system may be involved in the clinical efficacy of all antipsychotic drugs.

Despite the number of observed similarities NT shares with neuroleptics upon central injections, some differences are noted. For instance, in contrast to neuroleptics, NT neither interferes with the binding of radioligand to the dopamine receptors nor inhibits dopamine-stimulated adenylate cyclase activity (Nemeroff, 1980; Tamminga and Gerlach, 1987).

The purported role of NT as an endogenous neuroleptic has lead to the suggestion that NT may be implicated in syndromes characterized by dopamine imbalance, such as schizophrenia and Parkinson's disease. It was found that the cerebrospinal fluid (CSF) levels of NT were decreased in a subgroup of schizophrenic patients and that normal levels were resumed after a 1-month treatment with typical neuroleptics (Widerlov et al., 1982b). These results were later confirmed by other groups (Lindstrom et al., 1988; Nemeroff et al., 1989; for review see Kasckowet al., 1991). No changes in CSF NT levels have been noted in patients with other psychiatric disorders, including depression, Alzheimer's disease, anorexia-bulemia or premenstrual syndrome (Nemeroff et al., 1989). Recently, injection of a newly developed NT receptor agonist (PD149163) in a rat model of schizophrenia elicited antipsychotic effects that were distinct from those of both typical and atypical neuroleptics, supporting the view that centrally acting NT agonists may potentially be of use as antipsychotics (Feifel et al., 1999).

NT has also been implicated in a number of other neuromodulatory effects through its interaction with other neurotransmitter systems. It is a very potent stimulator of the hypothalamic-pituitary-adrenal axis activity; intracerebral injection of minute quantities

(μmol and pmol, icv) of the peptide rapidly results in a long lasting (>1h) increase in plasma ACTH levels (Rowe et al., 1992). Furthermore, chronic implants of the NT receptor antagonist SR48692 into the paraventricular nucleus of the hypothalamus decrease the activity of the hypothalamic-pituitary-adrenal axis under both basal and stress-induced conditions (Rowe et al., 1997) and chronic delivery of NT through intracerebroventricular injection leads to increased plasma ACTH levels, an effect reversed with SR48692. NT has also been implicated in the central regulation of the release of other pituitary hormones, including luteotrophic hormone, thyroid stimulating hormone, and growth hormone (for review see Rostene and Alexander, 1997).

Other non-neuroleptic effects of central NT-injections include reduced food intake in food-deprived rats (Luttinger et al., 1982b) and analgesia (Clineschmidt and McGuffin, 1977; Dubuc et al., 1994; Dubuc et al., 1999). Finally, NT appears to have the capacity to modulate both cortical activity and sleep-wake state. Its administration into the basal forebrain of freely moving rats stimulates cortical activation with rhythmic θ and γ waves independent of motor activity, in addition to promoting paradoxical sleep (Cape et al., 2000).

1.1.3 Distribution of neurotensin within the central nervous system

NT exhibits a widespread and heterogeneous distribution throughout the central nervous system. Initial radioimmunoassay studies carried out on rat brain extracts revealed that the highest concentrations of immunoreactive NT (iNT) are detected in the hypothalamus, bed nucleus of the stria terminalis, central nucleus of the amygdala, septum, substantia gelatinosa of the spinal cord and in the spinal trigeminal nucleus.

Moderate concentrations are detected in the accumbens nucleus, neostriatum, some thalamic nuclei, ventral tegmental area, periaqueductal grey, dorsal raphe, substantia nigra, and locus coeruleus. Very low concentrations of iNT are found in the cerebral cortex, hippocampus, and ventral portion of the spinal cord (Emson et al., 1982a).

Later, central iNT was shown by immunohistochemistry to be mainly concentrated in axons and axon terminals. NT-immunoreactive perikarya are relatively few in number. Within the rat brain, regions particularly rich in NT-containing fibres include the nucleus accumbens, bed nucleus of the stria terminalis, lateral septum, substantia innominata, globus pallidus, lateral and dorsal hypothalamic area, paraventricular nucleus of the hypothalamus, external zone of the median eminence, zona incerta, medial forebrain bundle, stria terminalis, mediodorsal, paraventricular and paracentral thalamic nuclei, habenular nuclei, medial and central nuclei of the amygdala, cingulate, retrosplenial and perirhinal cortices, substantia nigra, ventral tegmental area, interfascicular nucleus, and raphe nuclei (Emson et al., 1982b; Jennes et al., 1982; Emson et al., 1985a; Woulfe et al., 1994).

Visualization of NT-positive cell bodies requires pre-treatment of the animals with the microtubule disruptor colchicine (Emson et al., 1985b). In the rat brain, NT-immunoreactive cell bodies are particularly abundant in the septum, bed nucleus of the stria terminalis, amygdala and the arcuate and paraventricular nuclei of the hypothalamus. They are also present in lower concentrations in the olfactory tubercle, nucleus accumbens, neostriatum, discrete nuclei of the thalamus, preoptic area, periventricular hypothalamic nucleus, periaqueductal grey, substantia nigra, ventral tegmental area, locus coeruleus, and substantia gelatinosa of the spinal cord. Finally,

sparse NT cell bodies are detected in the diagonal band of Broca and ventral horn of the spinal cord (Emson et al., 1982b; Jennes et al., 1982; Emson et al., 1985a; Woulfe et al., 1994).

Combining NT immunohistochemistry with either retrograde transport of various markers or specific lesion studies has allowed the determination of several NT pathways in rat brain. These are summarized in Table 1.

In the adult rat brain, the distribution of NT mRNA as determined by *in situ* hybridization histochemistry (Alexander et al., 1989; Nicot et al., 1992) corresponds well with the distribution of NT-immunoreactive cell bodies as determined by immunohistochemistry (Emson et al., 1982b; Jennes et al., 1982; Emson et al., 1985a Woulfe et al., 1994), with a few exceptions. For instance, no iNT has been detected in the subiculum and CA1 region of the hippocampus despite the fact that a strong *in situ* signal has been reported in this region (Alexander et al., 1989). It was initially believed that this inconsistency was due to an incomplete maturation of the NT precursor molecule in the hippocampus. But this possibility was ruled out by the fact that NT, but not immature forms of the precursor, was detected by radioimmunoassay in the hippocampus (de Nadai et al., 1994). It was noted later that there was a drastic decrease in NT in the mammillary bodies (which receive projections from the subiculum) following section of the subiculo-mammillary tract (Nicot et al., 1995). Altogether, these results suggest that the absence of detectable iNT by immunohistochemistry in the subiculum may be due to a rapid transport of NT from its source in the subiculum to the mammillary bodies.

Other mismatches occured at the level of the dorsomedial part of the caudate/putamen and paraventricular and arcuate nuclei of the hypothalamus. In all of

Table 1: Neurotensin-containing pathways in the rodent brain

Originating cell bodies	*Projections*	*References*
Nucleus accumbens and lateral septum	Substantia innominata and medial preoptic area	(Morin and Beaudet, 1998)
Bed nucleus of the stria terminalis	Central nucleus of the amygdala, parabrachial nucleus	(Roberts et al., 1982; Moga et al., 1989)
Endopiriform nucleus and piriform cortex	Habenular nuclei, paraventricular and mediodorsal thalamic nuclei	(Inagaki et al., 1983b; Inagaki et al., 1983a)
Lateral septum and medial preoptic area	Anterior substantia innominata and diagonal band of Broca	(Morin and Beaudet, 1998)
Hippocampus	Frontal cortex	(Roberts et al., 1982)
Subiculum	Mammilary bodies	(Hara et al., 1982; Cuello et al., 1983; Kiyama et al., 1986)
Arcuate nucleus	Median eminence	(Hokfelt et al., 1984; Ibata et al., 1984b; Ibata et al., 1984a; Kiss et al., 1987)
Central nucleus of the amygdala	Bed nucleus of the stria terminalis, Substantia nigra, lateral	(Roberts et al., 1982; Vankova et al., 1992)
Substantia nigra	Caudate/putamen	(Zahm, 1989; Castel et al., 1990; Zahm et al., 1996)
Ventral tegmental area	Nucleus accumbens, medial olfactory tubercle, prefrontal cortex and amygdala	(Kalivas and Miller, 1984; Seroogy et al., 1987; Studler et al., 1988)
Central grey	Dorsal raphe magnus	(Beitz et al., 1983)
Nucleus of the solitary tract Central grey	Dorsal raphe	(Beitz, 1982)
Nucleus of the solitary tract	Parabrachial complex dorsal raphe, central amygdala	(Beitz, 1982; Milner and Pickel, 1986; Zardetto-Smith and Gray, 1990)

these areas, NT-immunoreactive cell bodies were numerous but NT/NN mRNA-expressing cells were scarce. These mismatches may be due to an ectopic production of iNT secondary to colchicine pre-treatment (Kiyama and Emson, 1991).

1.1.4 Ontogenesis of neurotensin

The ontogeny of NT expressing neurons has been studied using techniques of immunohistochemistry, radioimmunoassay and *in situ* hybridization. These studies have shown that NT neurons develop according to two distinct patterns. In certain neurons, NT mRNA is detected before birth, peaks during the postnatal period and continues to be detected during adult life (Sato et al., 1991). These neurons are found in the pyriform cortex, diagonal band of Broca, septum, bed nucleus of the stria terminalis, medial preoptic area, lateral hypothalamus, caudate/putamen, central nucleus of the amygdala, ventral tegmental area, dorsal raphe, laterodorsal tegmental nucleus and parabrachial nucleus. NT-immunopositive cells and fibers appear gradually, beginning at gestational day 16, peak at postnatal day 7 in the prosencephalon and diencephalon, and finally decrease to adult levels (Hara et al., 1982). In other neurons, NT mRNA is also detected before birth but is undetectable in adulthood (Sato et al., 1991). These neurons are restricted to the cingulate cortex, mitral cell layer of the olfactory bulb and rostral portion of the caudate/putamen. The role of this transitory mode of NT expression is poorly understood.

1.1.5 Neurotensin-related compounds: Neuromedin N, Large NT and Large NN

As previously stated, NT arises from the endoproteolytic maturation of a precursor molecule, pro-NT/NN. In addition to NT, the endoproteolytic maturation of this precursor can give rise to neuromedin N (NN) as well as to large forms of NT and NN (large NT and large NN, respectively). These peptides are endowed with biological properties distinct from those of NT.

NN is a hexapeptide (Lys-Ile-Pro-Tyr-Ile-Leu) which was isolated and characterized from porcine spinal cord on the basis of its gut contracting effect (Minamino et al., 1984). Like NT, NN appears also to act as a *bona fide* neurotransmitter in the CNS. The role of NN in the central nervous system has been much less investigated than that of NT, probably owing to the fact that NN's biological half-life is very short. Nonetheless, current data suggest that its effects are similar to those of NT. Accordingly, the last four amino acids of NN and NT are identical andNN was shown to bind to the same receptors as NT, albeit with different affinities (Checler et al., 1986a; Gaudriault et al., 1994).

Intracerebroventricular injection of NN in the mouse elicits NT-like effects, including hypothermia and analgesia (Coquerel et al., 1986; Dubuc et al., 1988). This peptide also increases locomotor activity and dopamine metabolism when injected in the ventral tegmental area of rats (Kalivas et al., 1986). It exerts the same effects as NT at equimolar amounts, but these effects are of shorter duration. This is attributed to the fact that NN gets inactivated more rapidly than NT by aminopeptidases (Checler et al., 1986a; Dubuc et al., 1988). Interestingly, there are instances in which NN was shown to be more potent than NT. For instance, when injected into the ventral tegmental area, NN was more potent than NT in increasing spontaneous motor activity and in increasing

dopamine metabolism in the nucleus accumbens, prefrontal cortex, diagonal band of Broca and septum. These data demonstrate that NN has a behavioral profile distinct from that of NT (Kalivas et al., 1986).

In the rat central nervous system, NT and NN exhibit similar distribution profiles, as determined by radioimmunoassay (Carraway and Mitra, 1987; de Nadai et al., 1989; Shaw et al., 1990; Kitabgi et al., 1991), although levels of NN are consistently lower than those of NT. The immunohistochemical distribution of E6I, a peptide sequence that is exposed only after NN has been cleaved from the precursor molecule (de Nadai et al., 1994), was also shown to parallel that of NT, suggesting that NN is co-expressed with NT in the rodent brain (Woulfe et al., 1994). In addition, the two peptides have been shown to be co-released following depolarization of mouse brain sections *in vivo* (Kitabgi et al., 1990). However, whether NT and NN are co-expressed at the cellular level remains to be determined.

Incomplete processing of the dual NT/NN precursor can lead to larger forms of NT and NN, namely large NT and large NN. Carraway and Mitra were the first to identify large NN as a peptide carrying the NN sequence at its C-terminal end (Carraway and Mitra, 1991). Sequencing of this larger form of NN later showed that it contained 125 amino acids corresponding to amino acids 24-148 of the precursor (Fig 1; Carraway et al., 1991). Large NN has been reported to be present only in minor concentrations in the dog (Carraway and Mitra, 1990), mouse (Shaw et al., 1990), and rat brain (de Nadai et al., 1994), but to be highly concentrated in the gut (Carraway and Mitra, 1990; Shaw et al., 1990; Carraway and Mitra, 1991; Carraway et al., 1991). The distribution of large NN has never been mapped out in mammalian brain.

Large NT starts after the signal peptide and ends with the NT sequence. It has never been detected in the brain but appears to be enriched in the adrenal medulla (Goedert et al., 1983; Fischer-Colbrie et al., 1992).

Since both large NT and large NN contain the NT and NN sequences at their C-terminus, it has been assumed that they interact with the same receptors as NT. However, little is known of their biological activity since they are too large to be chemically synthesized and their low level of expression makes their purification difficult. Nonetheless, in the periphery, large NN has been shown to induce the contraction of dog, cat and rat ileum with the same potency as NN (Carraway et al., 1991).

1.1.6 Organization of the neurotensin/neuromedin N gene

The NT/NN gene has been cloned and sequenced in the dog (Dobner et al., 1987), rat, cow (Kislauskis et al., 1988) and human (Bean et al., 1992). It is highly conserved across these species, especially in the region encoding NT and NN where it is 100% identical. The rat NT/NN gene coding regions is a 10.2 kilobase gene divided into 4 exons and 3 introns (Fig. 1).

-Exon 1: signal sequence

-Exon 2: sequences 23-44 of the precursor

-Exon 3: sequence 45-119 of the precursor

-Exon 4: sequence 120-169 of the precursor

The NT and NN coding domains are located in tandem on exon 4, near the carboxy terminus of the predicted precursor (Kislauskis et al., 1988). The promoter region of the gene is located 200 bp upstream of the TATA box. This region contains several

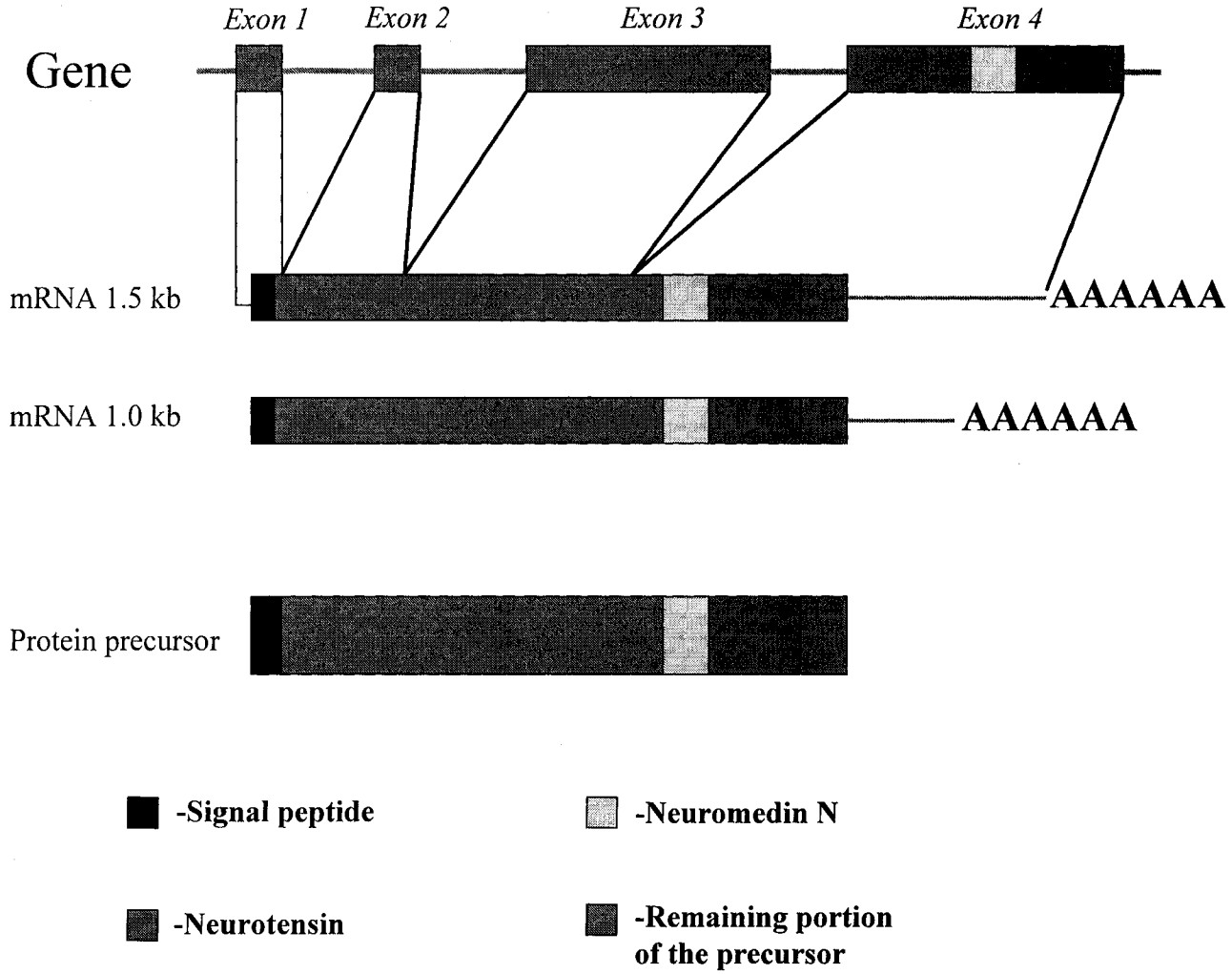

Figure 1: ***Schematic representation of the organization of the gene, mRNA and protein of prepro-NT/NN.*** The prepro-NT/NN gene is composed of 4 exons. NT and NN both arise from exon 4 and the remaining segments of the precursor from exons 1 to 3. Note that two mRNA are generated from the gene. They differ only in their degree of polyadenylation (from Rovère, 1996).

inducible *cis*-regulator sequences: An AP-1 sequence, a cAMP response element (CRE), a glucocorticoid regulatory element (GRE), and a sequence, which is both CRE and AP-1 (Kislauskis et al., 1988; Kislauskis and Dobner, 1990; Dobner et al., 1992).

The AP-1 sequence is recognized by the proto-oncogenes *c-fos* and *c-jun.* The CRE sequence is recognized by a homodimer of the phosphorylated form of the cAMP response element binding-protein. The GRE is recognized by a homodimer formed by two glucocorticoid receptors which could be activated by cortisol. The AP-1 and CRE sequences act as activators of the NT/NN gene, while the GRE only plays a permissive role (Augood and Emson, 1992).

The NT/NN gene generates two transcripts: one of 1.0 Kb and the other of 1.5 Kb (Fig. 1; Kislauskis et al., 1988). The difference in size between these two forms of mRNA is due to the existence of two polyadenylation sites, the 3' end of the 1.5 Kb mRNA being 400 bases longer than that of the 1.0 Kb transcript. Whereas the 1.0 Kb mRNA greatly predominates in the intestine (Kislauskis et al., 1988) and in the anterior pituitary (Jones et al., 1989), approximately equal amounts of 1.0 kb mRNA and 1.5 kb mRNA are present in the brain (Kislauskis et al., 1988).

Since NT and NN are located in the same exon at the gene level, this leads to the suggestion that the mRNA will encode for a common peptide precursor molecule. Thus, the two peptides will possibly be co-released.

1.1.7 Pro-neurotensin/neuromedin N polypeptide; a precursor that undergoes differential processing

The NT/NN gene encodes Pro-NT/NN, a 169-170 amino acid precursor containing NT and NN positioned in tandem near the carboxy-terminus. In addition, a sequence resembling NN (NN-like) occurs in the central region of the NT/NN precursor (Fig. 2). Pro-NT/NN contains four Lys-Arg sequences (Lys^{85}-Arg^{86}, Lys^{140}-Arg^{141}, Lys^{148}-Arg^{146}, Lys^{162}-Arg^{163}), which represent potential sites for proteolytic cleavage by enzyme members of the convertase family (Fig. 2; Muller and Lindberg, 1999; Seidah et al., 1999a; Bergeron et al., 2000). Differential cleavage at these pairs of basic residues has been shown to yield different combinations of maturation products, depending on the tissues in which the NT/NN precursor is being processed. In the gut, the two most C-terminal basic residues are processed preferentially, giving rise to comparable amounts of NT and large NN (Fig. 2; Carraway et al., 1990, 1991). In the brain, the three most C-terminal pairs of basic residues are extensively processed, thus releasing roughly equimolar amounts of NT and NN, whereas the most N-terminal pair of basic residues site (Lys^{85}-Arg^{86}) is considerably less efficiently cleaved (de Nadai et al., 1994; Woulfe et al., 1994). Comparison of iNT and iNN concentrations from microdissected brain areas in the rat brain reveals that the ratio of the former to the latter varies significantly from one brain region to the next, ranging from 0.4 to 4.5 (de Nadai et al., 1989; Kitabgi et al., 1991). The highest NT/NN ratios are found in the globus pallidus, posterior hypothalamus and dopaminergic regions such as the substantia nigra and ventral tegmental area, whereas values close to 1 are found in cortical regions, in the striatum, hippocampus and mammillary body. The observation of regional variations raises the possibility of region-specific differences in NT/NN precursor processing.

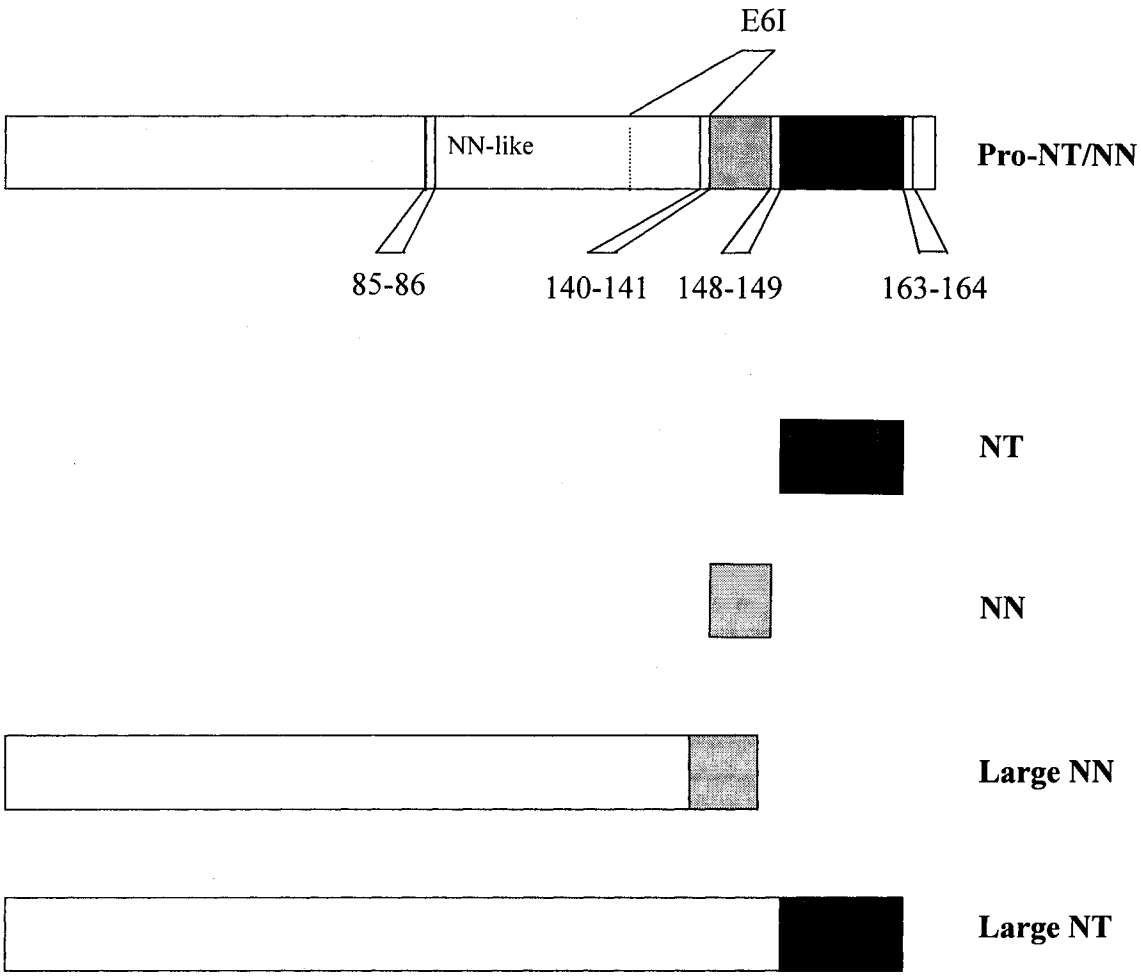

Figure 2: ***Schematic representation of pro-NT/NN and its various maturation products.*** The position of the four Lys-Arg dibasic sites are illustrated along with NT, NN, large NN, large NT and the peptide segment E6I exposed after proteolytic cleavage of the Lys^{140} - Arg^{141} dibasic site.

Large NN is also detected in the brain and regional variations in its concentration relative to NT and NN have been reported (de Nadai et al., 1994).

1.1.8 Neurotensin/neuromedin N receptors

NT and NN exert their actions through interaction with multiple cell membrane receptors. Initial evidence for the existence of NT receptors was derived from radioligand binding experiments on membranes prepared from brain and gastrointestinal tissues (Kitabgi et al., 1977). Pharmacological studies later suggested the existence of at least two different NT receptor sub-types, one with high affinity for NT (Kd = 0.2-0.3 nM) and sensitive to the non-peptidic NT antagonist SR48692 (Mazella et al., 1983) and another with a lower affinity for NT (Kd = 2-4 nM; Schotte et al., 1986) and sensitive to the antihistamine levocabastine (Schotte et al., 1986; Kitabgi et al., 1987). Both of these receptors have been cloned from mouse, rat and human (Tanaka et al., 1990; Vita et al., 1993; Chalon et al., 1996; Mazella et al., 1996; Botto et al., 1997b; Snider et al., 1998). They are referred to as NTS1 and NTS2, respectively, and both belong to the G protein-coupled seven transmembrane domain receptor family, characteristic of biogenic amines and of most neuropeptide receptors.

The NTS1 receptor has been linked to a variety of signalling cascades, including stimulation of cGMP production, turnover of inositol phosphates and Ca^{++} mobilization (Amar et al., 1985; for review see Vincent, 1995). In addition to NT, NTS1 binds to the other pro-NT/NN maturation products, albeit each one with a distinct affinity: NN bind to NTS1 with an IC50 of 3 nM. The affinity of large NT closely resembles that of NT, with an IC50 of 1.9 nM (vs 0.45 for NT) whereas that of large NN is significantly less

than that of NN (40.8 nM vs 3.0 nM; Feliciangeli et al., personal communication).

Experiments using the selective NTS1 antagonist SR48692 have shown that in the central nervous system, NTS1 is involved in the regulation of midbrain dopaminergic (Gully et al., 1993; Poncelet et al., 1994; Gully et al., 1997) and forebrain cholinergic (Alonso et al., 1994; Steinberg et al., 1995; Cape et al., 2000) neurons, as well as in the modulation of the hypothalamic-pituitary-adrenal axis (for review see Rostene and Alexander, 1997). Accordingly, immunohistochemical studies using antibodies specific for NTS1 (Boudin et al., 1996), autoradiographic studies using ^{125}I-NT in the presence of blocking concentrations of levocabastin (to prevent labeling of the low affinity receptor), and *in situ* hybridization studies using specific mRNA oligonucleotide probes have revealed the presence of high concentrations of NTS1 in association with midbrain dopaminergic (Szigethy and Beaudet, 1989) and basal forebrain cholinergic (Szigethy and Beaudet, 1987) neurons as well as with various hypothalamic and limbic structures (Moyse et al., 1987; Elde et al., 1990; Nicot et al., 1994; Boudin et al., 1996; Alexander and Leeman, 1998). At the cellular level, NTS1-immunoreactivity was predominantly associated with perikarya and dendrites in some regions (e.g., in the basal forebrain, ventral midbrain, pons and rostral medulla) and with axons and axon terminals in others (e.g., in the lateral septum, bed nucleus of the stria terminalis, neostriatum, paraventricular nucleus of the thalamus and nucleus of the solitary tract), suggesting that NTS1 may act both post-and presynatptically in the central nervous system (Boudin et al., 1996).

The NTS2 receptor is also a G protein-coupled receptor and its interaction with NT leads to different transductional effects, depending on the cell model used and the species

under study. For instance, when the receptor is expressed in Xenopus oocytes, both NT and NN are able to trigger an inward Ca^{2+} current (Botto et al., 1997a) whereas such is not the case in CHO cells transfected with human NTS2 (Vita et al., 1998). Recently, it was found that NT induces a MAP kinase phosphorylation response but no Ca^{2+} currents in rat cerebellar granule cells endogenously expressing NTS2 (Sarret et al., 2001), confirming that NT acts as agonist on NTS2 expressed in neurons. The interaction of NTS2 with the other pro-NT/NN maturation products has not been investigated to date. The functional role of NTS2 is still unclear. However, there is a growing body of evidence to suggest that it might be responsible for the mediation of the antinociceptive effects of NT. Thus, NT-induced antinociception is not antagonized by the NTS1 antagonist SR48692, but is blocked by SR142948, which recognizes both NTS1 and NTS2 (Gully et al., 1997). Accordingly, NT-analgesic effects are abolished *in vivo* after the injection of NTS2 antisense oligonucleotides against NTS2 (Dubuc et al., 1999). The distribution of NTS2 is consistent with its proposed mediation of NT-induced analgesia since NTS2 mRNA, as well as NTS2 binding sites, are enriched in brainstem structures implicated in descending antinociceptive influences (e.g., the periaqueductal gray, nucleus raphe magnus, gigantocellular reticular nucleus, pars alpha, and lateral paragigantocellular nucleus; Kitabgi et al., 1987; Sarret et al., 1998; Walker et al., 1998). In addition to its purported role in antinociception, NTS2 is likely implicated in the modulation of primary afferent pathways since its mRNA is predominantly expressed in major sensory afferent systems (Sarret et al., 1998).

At the cellular level, NTS2 is expressed by both neurons and glial cells (Sarret et al., 1998; Walker et al., 1998). The glial expression of NTS2 is low under basal conditions

but is massively up-regulated during astrocytic reaction, suggesting that it may play a role in regulating glial response to injury (Nouel et al., 1999). The subcellular distribution of NTS2 has not been investigated to date.

A third NT receptor has been cloned (NTS3; Mazella et al., 1998) and found to correspond to sortilin, a type 1 membrane protein with a single transmembrane domain (Petersen et al., 1997). It binds NT with an affinity close to that of NTS1 and, in fact, had been originally affinity-purified as a 100 kDa NT receptor protein (Mazella et al., 1998). High levels of NTS3 expression were reported in the brain, spinal cord, heart, skeletal muscle, and testis (Petersen et al., 1997). NTS3 cell surface expression is low under unstimulated conditions and it appears to be mainly associated with the Golgi apparatus and intracellular vesicles. However, upon down-regulation of the NTS1 and NTS2 receptors, NTS3 is translocated to the cell surface (Mazella et al., 1998). Whether the NTS3 represents a true NT receptor and what role this receptor may play in the brain remains to be elucidated. In the periphery, NTS3 was shown to be expressed by human cancer cells documented to be sensitive to the trophic effects of NT, suggesting that this receptor subtype might be involved in the growth promoting activity of NT (Dal Farra et al., 2001).

1.1.9 Release and mechanisms of inactivation of neurotensin and neuromedin N

NT and NN are jointly released into the synaptic cleft upon neuronal depolarization. In the synaptic cleft, both peptides are degraded through the combined action of endo- and amino-peptidases. NT is inactivated through the action of metallo-endopeptidases 24.11 (E24.11; enkephalinase), E24.15 (thimet oligopeptidase) and E24.16 (neurolysin).

These peptidases jointly cleave the biologically active C-terminus of NT, thereby rendering it inactive (Checler et al., 1986c; Checler et al., 1986b; Checler et al., 1987; Barelli et al., 1989). NN is primarily inactivated through the removal of its amino-terminal Lys residue by aminopeptidases B and M (for review see; Checler, 1994). Other exo- and endo-peptidases not described hereinafter participate in the further degradation of the breakdown products generated by the metallo-endopeptidases (Checler et al., 1985; Checler et al., 1986c; Checler et al., 1986b; Checler et al., 1987; Barelli et al., 1989). Since aminopeptidases are abundant in all tissues, NN is usually more rapidly degraded than NT, the half-life of NN being about 2.5 times shorter than that of NT in rat brain synaptic membranes (Checler et al., 1986a). These peptidases are likely to be physiologically relevant since co-injection of NT or NN with specific peptidase inhibitors leads to an enhancement of both NT and NN effects. For example, intracerebroventricular injection of NT with thiorphan, a potent and specific endopeptidase 24.11 inhibitor, markedly potentiated the hypothermic and analgesic effects of NT (Coquerel et al., 1986; Dubuc et al., 1988), whereas co-injection of the aminopeptidase inhibitor bestatin did not significantly affect the NT-induced responses but enhanced the hypothermic and analgesic effects of NN injected intraventricularly in the mouse (Dubuc et al., 1988). Furthermore, there is evidence for regional variations in the catabolism of NT and NN in both brain and gut, probably due to differential expression of these various endopeptidases (Checler et al., 1991; Checler, 1994). This differential inactivation may in turn affect the physiological responses to NT and NN in a tissue-specific manner.

Interestingly, when large NT and large NN were assayed for their stability, it was

found that both possess an enhanced stability relative to their corresponding fully processed forms, suggesting that they might induce longer cellular responses and/or act at a longer distance from their point of release than their corresponding shorter forms (Feliciangeli et al., *submitted*).

Not all of the released NT is degraded in the extracellular space since a portion of the released NT is internalized into its target cells via a receptor-mediated process (Mazella et al., 1991; Beaudet et al., 1994). Simultaneous visualization of both fluo-NT and NTS1 following NT-induced internalization revealed that fluo-NT was targeted intact to the TGN and may possibly be recycled back to the extracellular space, whereas NTS1 was predominantly targeted to lysosomes for degradation (Vandenbulcke et al., 2000). By contrast, NTS2 is recycled back to the plasma membrane following ligand-induced internalization (Botto et al., 1998; Nguyen et al., *submitted*). The physiological significance and the mechanisms regulating this process are being currently investigated. It is thought that, in addition to down-regulating cell surface receptors, the internalization process may mediate transcriptional effects in a G-protein independent manner (Souaze et al., 1997).

1.1.10 Biological activity of pro-neurotensin/neuromedin N-derived products is regulated at several levels

In the previous sections, we have reviewed the different steps involved in the regulation of pro-NT/NN-derived products synthesis and activity. It becomes apparent that, in order to understand the physiology of pro-NT/NN producing and target systems,

it is necessary to consider the dynamics of the mechanisms that govern pro-NT/NN maturation.

1.2 MECHANISMS OF PEPTIDE BIOSYNTHESIS

Biosynthesis of peptide neurotransmitters involves initial synthesis of large precursor molecules, pre-proproteins, that are subsequently modified along the regulated secretory pathway.

Modifications of the peptide precursor start co-translationally and, in most cases, continue after translation is finished. In the rough endoplasmic reticulum (RER), the signal peptide is cleaved by the signal peptidase and the proprotein undergoes N-linked glycosylation, disulfide bond formation, and acquisition of its tertiary structure through the folding of the α-helices, β-sheets, and β-turns. Propeptides are transferred from the RER to the cis-Golgi element and then from one Golgi stack to the next via transfer vesicles. During transit in the Golgi apparatus, peptide precursors undergo a series of modifications, including O-linked glycosylation, tyrosine sulfation, serine sulfanation, serine phosphorylation, N-acetylation (Kornfeld and Kornfeld, 1985), and selective endoproteolysis at specific consensus sequences (Rothman and Orci, 1992).

Proteolytic processing usually takes place at the carboxy-terminal side of paired basic amino acid residues, Lys-Arg (Fig. 3) and Arg-Arg, or at a motif of basic residues whose consensus sequence can best be represented by (Lys/Arg)-$(X)_n$-(Lys/Arg)↓, where n = 0, 2, 4 or 6 and X is any amino acid and is usually not cysteine (Seidah and Chretien, 1999). Less commonly, cleavage can also occur at sites containing one, three, or four basic amino acid residues or even at non-basic residues (Hudson et al., 1981; Jornvall et

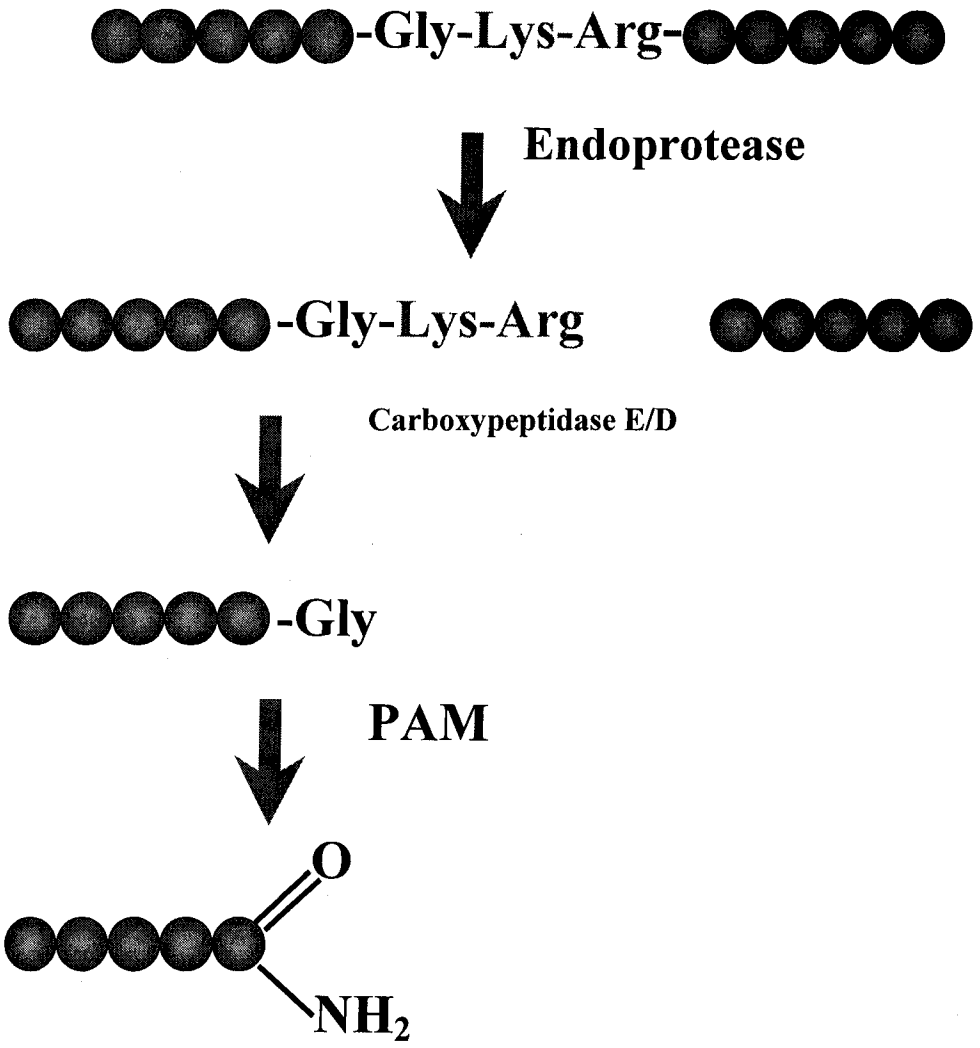

Figure 3: ***Steps in endoproteolytic maturation.*** Endoproteolysis usually takes place at the carboxy-terminal side of the recognition site, usually consisting of a pair of basic residues. This is followed by trimming of the basic residues by carboxypeptidase E. If the peptide ends with glycine, the enzyme peptidyl-glycine-α-amidating monooxygenase (PAM) acts, leaving an amide on the carboxyl terminus of the peptide (Modified from Sossin, 1989).

al., 1981). Cleavage at nonbasic residues (e.g. pro-gastric inhibitory peptide and pro-relaxin) has been documented, precluding any definitive consensus cleavage site (Hudson et al., 1981; Jornvall et al., 1981). Furthermore, some pairs of basic residues residues and most single basic residues contained within a peptide precursor are not cleaved, suggesting that the conformation of the surrounding protein sequence also plays a role in determining the site of cleavage. In this context, it has been suggested that the cleavage itself may require the basic residues to be positioned in, or immediately adjacent to, a β-turn (Rholam et al., 1986). Another level of complexity is added by the fact that cleavage of processing sites may vary in a tissue-specific, or even in a region-specific manner. This is well illustrated by the region-specific maturation profile of the multifunctional precursor pro-opiomelanocortin (POMC) which matures into ACTH and β-lipotropin in cells of the anterior pituitary and into α-melanocyte-stimulating hormone, γ-lipotropin and β-endorphin in the intermediate lobe (Benjannet et al., 1991). Such tissue-specific maturation has been reported for several other peptide precursors including pro-neurotensin/neuromedin N (Kitabgi et al., 1991; de Nadai et al., 1994; Woulfe et al., 1994), pro-somatostatin (Sawchenko et al., 1988), pro-dynorphin (Zamir et al., 1994), pro-thyrotrophin-releasing hormone (Bulant et al., 1988), and pro-enkephalin (Zamir et al., 1984; Day and Akil, 1989). In addition, developmentally regulated processing of glucagon has been reported in the brain (Lui et al., 1990).

In almost all instances, at least some of the peptides generated by propeptide conversion have no known biological function as secretory products. A number of roles have been attributed to these peptide segments retained within the cleaved precursor molecule, including a role in the targeting of the processing products to the regulated

pathway. Thus, the proregion of pro-somatostatin was shown to be important for the proper targeting of somatostatin to the regulated secretory pathway (Stoller and Shields, 1989) and the connecting peptide (C-peptide) of pro-insulin was demonstrated to be necessary for proper pro-insulin conversion (Markussen and Schiff, 1973; Gross et al., 1989). The processed precursor proregions have also been postulated to help the precursor fold into its correct three-dimensional structure or to serve as a spacer peptide to assure the minimum length necessary for a pre-proprotein to penetrate into the lumen of the RER. Alternatively, they could also be endowed with as yet unidentified biological properties on their own. For instance, it was recently found that intravenous injection of the C-peptide contained within pro-insulin prevents diabetes- and hyperglycemia-induced vascular and neural dysfunction in animal models of diabetes. As such, it is now considered by some as an adjunct agent in the management of diabetes (Ido et al., 1997).

Endoproteolysis is followed, in some cases, by the action of carboxypeptidases (e.g. carboxypeptidases D and E) to remove residual arginine and lysine residues (Fricker, 1988; Song and Fricker, 1995). This is followed, in the case of amidated peptides, by conversion of the carboxyl-terminal glycine into an amide by the amidating enzyme (PAM; peptidyl glycine α-amidating mono-oxygenase; Fig. 3; Eipper et al., 1983). Endoproteolysis is usually initiated in the trans-Golgi network (TGN) but the bulk of this activity reportedly occurs in the dense core granules for most propeptides (Gainer et al., 1985). However, there are examples of endoproteolysis taking place as early as within the Golgi apparatus (e.g. pro-thyrotropin releasing hormone; Nillni et al., 1993), pro-somatostatin (Lepage-Lezin et al., 1991) and pro-egg-laying hormone (Sossin et al.,

1990a). Such early processing could lead to the independent sorting of various portions of a prohormone, as it has been shown to occur for Aplysia's pro-egg-laying hormone (Sossin et al., 1990b). Conversely, cleavage after packaging into dense core vesicles ensures the co-existence and co-release of a set of peptide products. The subcellular compartment in which the endoproteolytic cleavage takes place is therefore critical in the case of multifunctional precursors.

In addition to their evident role in the endoproteolytic cleavage of precursor molecules, pairs of basic residues residues contained at the cleavage sites could play a determinant role in the sorting of at least some propeptide molecules. Thus, point mutation at pairs of basic residues residues normally processed in either pro-renin or pro-somatostatin prevented their routing to the regulated secretory pathway (Brakch et al., 1994; Brechler et al., 1996). More recently, Feliciangeli et al. (2001) showed that point mutations at all three pairs of basic residues sites within the C-terminal tail of pro-NT/NN abolished the regulated secretion of its maturation products.

The sorting of peptides to the regulated pathway of secretion occurs in the TGN. Mechanisms responsible for this sorting are still controversial, and may be either active, involving specific receptors at the level of the TGN, or passive, such that constitutively secreted proteins would be removed from vesicles targeted to the regulated secretory pathway (Orci, 1982; Arvan and Castle, 1998; Glombik and Gerdes, 2000). Either way, neuropeptides are concentrated in secretory vesicles and routed to their site of secretion. Mature forms of the peptides are then released upon appropriate stimulation.

The differential endoproteolysis of a given peptide precursor is likely to be physiologically relevant since the generated mature peptides often activate a diverse set

of receptors to elicit distinct or even opposite effects. Therefore, the enzymes responsible for the processing of peptide precursors play a key role since, in fact, they modulate the bioactivity of the neuropeptides.

1.2.1 Discovery of the prohormone convertases

The concept that peptides arise from the endoproteolytic maturation of larger precursor molecules was first proposed almost simultaneously by Steiner (1967) and Chrétien and Li (1967) on the basis of experimental procedures derived from different approaches. Steiner and co-workers characterized the structure of the peptide precursor pro-insulin and found that exposure of purified tissue extract containing these large molecules to small amounts of trypsin led to an increase of mature insulin (Steiner, 1967). Chrétien and Li determined the structures of β-lipotropin (β-LPH), γ-LPH and β-MSH and observed that β-MSH sequence was embedded in the sequences of both β-LPH and γ-LPH. They concluded that β-MSH was a product of proteolytic processing of the other two hormones (Chretien and Li, 1967).

The first enzyme identified as being able to cleave peptide precursors was discovered in the mid-80s using yeast genetics and corresponded to Kex2. This enzyme was shown to be a Ca^{2+}-dependent serine protease with a subtilisin/kexin-like catalytic domain. It is necessary for the cleavage of the carboxyl side of paired residues in the pro-α-mating factor of yeast (Julius et al., 1984) and is able to properly process mammalian peptide precursors *in vitro* (Thomas et al., 1988). Since then, eight mammalian prohormone convertases (PC) have been identified on the basis of their homology with Kex2. All PCs of this family are Ca^{2+}-dependent serine proteases with a highly

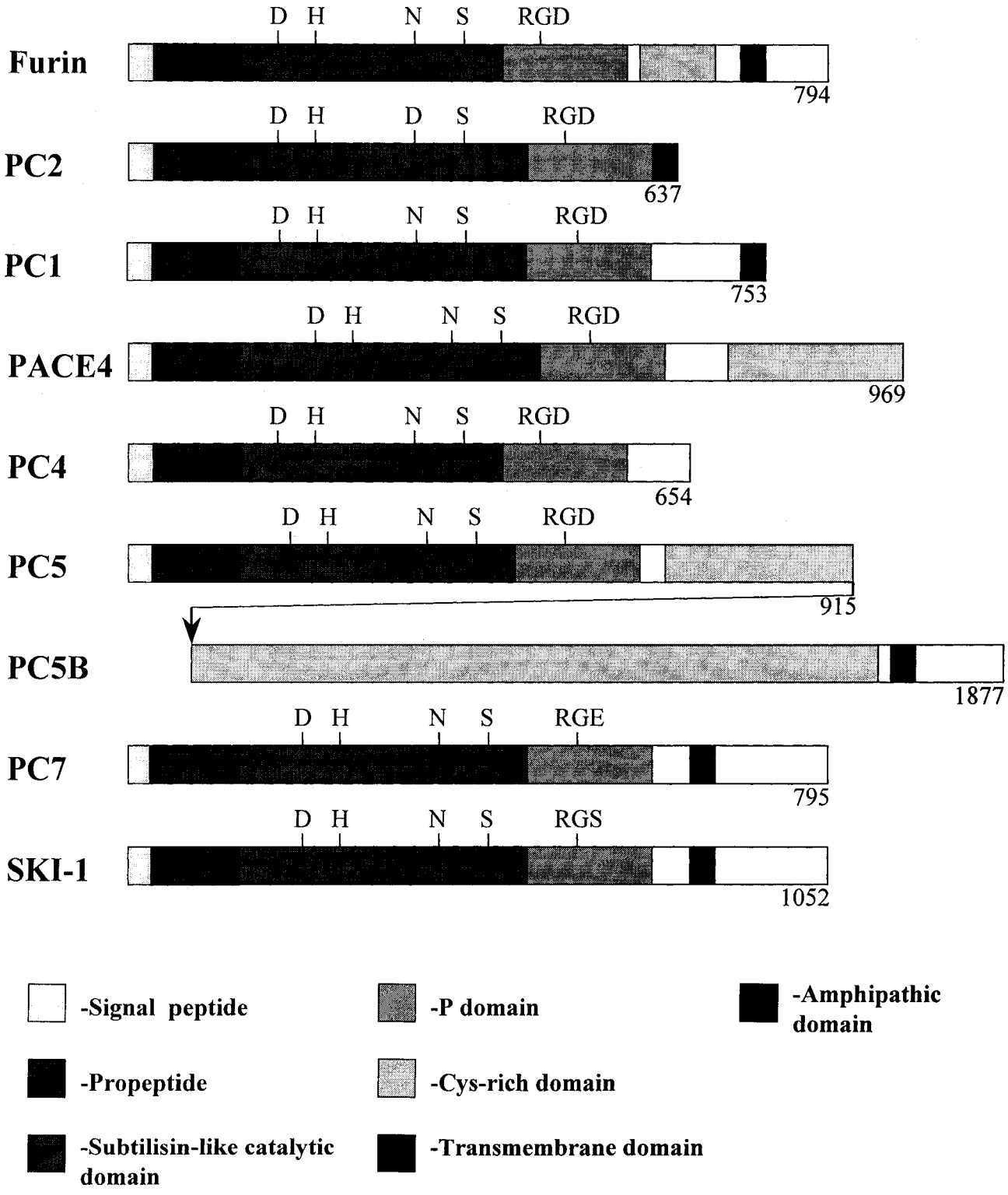

Figure 4: ***Schematic representation of the convertases.*** The catalytic Asp (D), His (H), Ser (S) and Asn (N)(Asp for PC2) residues and the Arg-Gly-Asp (RGD; RGS for PC8) sequence are shown. The number of amino acids are indicated for each protease.

conserved catalytic domain. These are referred to as PC1 (Seidah et al., 1990; Seidah et al., 1991b) (also known as PC3; Smeekens et al., 1991), PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990; Ohagi et al., 1992), furin (Roebroek et al., 1986; Fuller et al., 1989; Barr et al., 1991), PACE4 (Kiefer et al., 1991), PC4 (Nakayama et al., 1992; Seidah et al., 1992a), PC5/6A (Lusson et al., 1993; Nakagawa et al., 1993), PC7 (Seidah et al., 1996b), also known as PC8 (Bruzzaniti et al., 1996) or LPC (Meerabux et al., 1996) and SKI-1 (Seidah et al., 1999b) (Fig. 4; for review see; Seidah et al., 1997, 1999; Bergeron et al., 2000). In addition, protein convertase isoforms resulting from the differential splicing of the mRNA precursor exist for PC5 (Nakayama et al., 1992), PACE4 (Mori et al., 1997; Tsuji et al., 1997), PC4 (Nakayama et al., 1992; Seidah et al., 1992a) and SKI-1 (Seidah et al., 1999b). Enzymatic activity has been associated only with the two PC5 isoforms, PC5A and PC5B (Nakagawa et al., 1993; Seidah et al., 1996a; Laframboise et al., 1997; Barbero et al., 1998).

1.2.2 Distribution and function of the convertases

The prohormone convertases can be broadly divided into four classes, based on their tissue distribution, function and intracellular localization: 1) Furin and PC7 exhibit a rather ubiquitous tissue distribution and play a role in the maturation of precursors routed through the constitutive pathway of secretion. Furin is particularly enriched in liver and kidney (Schalken et al., 1987) whereas PC7 is most highly expressed in lymphoid-associated tissues (Seidah et al., 1996b). At the intracellular level, they are both concentrated at the level of the TGN (Fig. 5; Molloy et al., 1994; de Bie et al., 1996). Proproteins processed by furin and PC7 include growth factors, serum proteins (including

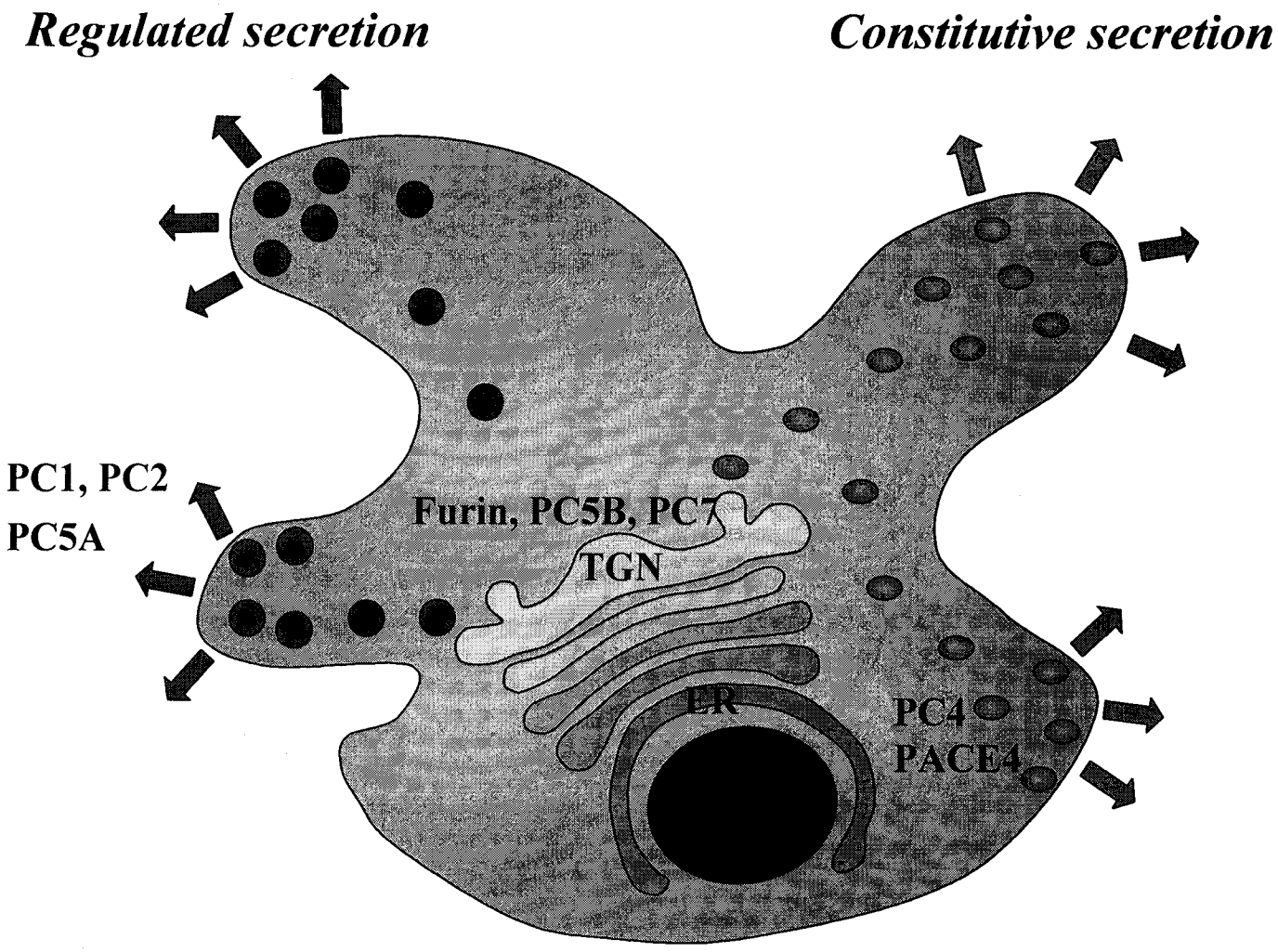

Figure 5: ***Schematic representation of the cellular localization of the PCs.*** PC1, PC2 and PC5A are sorted to dense core vesicles (green circles), within the regulated secretory pathway of secretion. In contrast, the membrane-bound furin, PC5B and PC7 are mostly localized within the TGN. Although the localization of PACE4 and PC4 is not yet determined, these enzymes appear to be secreted through the constitutive secretory pathway (Modified from Seidah and Chrétien, 1997).

proteases of the blood-clotting and complement systems), matrix metalloproteinases, receptors, viral-envelope glycoproteins, and bacterial exotoxins (for review see; Nakayama, 1997; Seidah and Chretien, 1997); 2) PACE4 and PC5B are expressed in both endocrine and non-endocrine cells (Kiefer et al., 1991; Lusson et al., 1993; Nakagawa et al., 1993; Dong et al., 1995) and both appear to possess the ability to process precursors routed to both the constitutive and regulated secretory pathway. The subcellular distribution of PACE4 is isoform-dependent, whereas PC5B is concentrated at the level of the TGN (De Bie et al., 1996; Mori et al., 1997); 3) PC4 is exclusively expressed in reproductive glands (Nakayama et al., 1992; Seidah et al., 1992a; Torii et al., 1993). Its subcellular distribution remains to be determined and, to date, it has only been shown to process the pituitary adenylate cyclase-activating polypeptide precursor (PACAP; Li et al., 2000); 4) PC1 and PC2 are expressed exclusively by endocrine and neuronal cells and both process peptide precursors routed to the regulated secretory pathway (Fig. 5; Lindberg, et al., 1994; Rovere et al., 1996). The highest levels of expression of these two PCs are found in the brain (Schafer et al., 1993; Winsky-Sommerer et al., 2000), but they are also present in the thyroid gland, pancreas, adrenal gland, and gut (Seidah et al., 1991a; Seidah et al., 1991b; Smeekens et al., 1991). Propeptides processed by either one or both of these convertases *in vitro* include pro-enkephalin (Mathis and Lindberg, 1992; Breslin et al., 1993; Johanning et al., 1996), pro-somatostatin (Brakch et al., 1995; Galanopoulou et al., 1995), pro-thyrotropin-releasing hormone (Schaner et al., 1997), POMC (Benjannet et al., 1991; Bloomquist et al., 1991; Thomas et al.., 1991; Seidah et al., 1992b; Friedman et al., 1994), pro-thyrotropin-releasing hormone (Friedman et al., 1995), pro-NT/NN (Rovere et al., 1996a), pro-

dynorphin (Dupuy et al., 1994), pro-neuropeptide Y (Paquet et al., 1996; Brakch et al., 1997), pro-cholecystokinin (Wang et al., 1998), and pro-melanin concentrating hormone (Viale et al., 1999). Evidence suggests that PC5A is also specific for the regulated secretory pathway, although this remains to be confirmed (De Bie et al., 1996). PC5A is particularly abundant in the intestine, adrenals, and the brain (Lusson et al., 1993; Nakagawa et al., 1993).

SKI-1 is the most recently cloned convertase and it possibly belongs to a new family of subtilisin-kexin-isozymes since it cleaves precursors at single (and possibly pairs of) hydroxylated amino acids, such as threonine (Seidah et al., 1999b). SKI-I mRNA is very widely, if not ubiquitously. distributed, showing high expression in the thymus, adrenal, submaxillary gland, and pituitary (Seidah et al., 1999b).

1.2.3 Structure of the prohormone convertases

Prohormone convertases are highly conserved among the eukaryotic proprotein convertase family. All known PC proenzymes possess a signal peptide, a prosegment, a highly conserved catalytic domain, a segment referred to as the P-domain and an enzyme-specific C-terminal segment (Fig. 4) The prosegment is thought to act as a molecular chaperone guiding the folding of the PCs in the ER (Eder et al., 1993; Shinde and Inouye, 1993; Lesage et al., 2000). The catalytic domain of each convertase is the most conserved of all domains. In particular, the catalytic triad residues Asp, His and Ser, and an Asn residue are present in concordant positions in all convertases except PC2 (Fig. 4). These residues stabilize the oxyanion hole in the transitional state (Bryan et al., 1986). In PC2, Asp replaces the Asn residue. The significance of this residue is unclear since an

Asp → Gln mutation at this site has been reported to produce only minor effects on PC2 enzymatic activity (Zhou et al., 1995a). The P-domain appears to be important for the folding of the proteinase and for the elaboration of its full enzymatic activity (Lipkind et al., 1998; Zhou et al., 1998). All PCs discovered so far, with the exception of PC7, contain within their P-domain a conserved Arg-Gly-Asp sequence that is reminiscent of the recognition sequence for integrins (Fig. 4). The function of this sequence is still unknown, although mutations of either one of these three residues in PC1 impairs its intracellular sorting to secretory granules as well as its stability within the ER (Lusson et al., 1997; Rovere et al.,1999).

The C-terminal portion of the PCs is the segment of the enzyme exhibiting the greatest degree of variability. PC5A and PC5B isoforms differ at the level of their C-terminus, PC5B being a C-terminal extended isoform of PC5A (Nakagawa et al., 1993). Furin (Roebroek et al., 1986), PC5B (Nakagawa et al., 1993), PC7 (Seidah et al., 1996b) and SKI-1 (Seidah et al., 1999b) have transmembrane domains, which, in the case of furin and PC5B, have been shown to anchor the enzyme to the membrane of the TGN (Molloy et al., 1994; Schafer et al., 1995; De Bie et al., 1996). PC1 and PC2 exhibit an amphipathic C-terminal sequence which is postulated to interact with membranes along compartments of the secretory pathway of secretion in a pH-dependent manner (Fricker et al., 1990). Finally, furin, PACE4, and both PC5 isoforms exhibit a C-terminal cysteine-rich region, the function of which is currently unknown.

The C-terminal domain contained within the PCs could play a role in the sorting of proteases to the regulated secretory pathway since the deletion of C-terminal domain residues in PC5A increases the secretion of this enzyme through the constitutive pathway

(De Bie et al., 1996). However, opposing results are obtained in the case of PC1 since a PC1 mutant truncated at its C-terminus was shown to be efficiently routed to the secretory pathway and stored in secretory granules, indicating that the carboxyl-terminal extension is not required for the sorting of this enzyme (Zhou et al., 1995b).

1.2.4 Post-translational processing of the convertases

PCs are themselves synthesized as zymogens that undergo endoproteolytic processing. Furin, PC1, PC5 and PC7 have been shown to undergo autocatalytic intramolecular processing of their N-terminal profragment (Leduc et al., 1992; Benjannet et al., 1993; Lindberg et al., 1994; Zhou and Mains, 1994; De Bie et al., 1995; Munzer et al., 1997). Proteolytic removal of the proregion occurs in the ER and is essential for enzyme activation (Leduc et al., 1992). Indeed, mutations of the proregion cleavage site prevent full activation of Kex2 and PC1 (Goodman and Gorman, 1994; Lesage et al., 2000) and lead to the accumulation of an inactive form of furin in the ER (Leduc et al., 1992). Moreover, deletion of the proregion inactivates furin (Leduc et al., 1992). These results suggest that the presence of a cleavable prosequence is necessary to produce active enzyme and for proper subcellular trafficking of the enzyme. This is consistent with a role of PC proregions in the folding of the mature protease domain. Furthermore, it appears that, at least in the case of furin, PC1, and PC7, the proregion behaves as a transient antoinhibitor of enzymatic activity until it is disposed of during cellular transit of the enzyme to the TGN (Leduc et al., 1992; Boudreault et al., 1998; Zhong et al., 1999). In contrast to the zymogen precursors of the other PCs, pro-PC2 cleavage is much slower since pro-PC2 is retained in the RER and only gives rise to mature PC2 within the

TGN (Benjannet et al., 1993; Shen et al., 1993; Zhou and Mains, 1994). Pro-PC2's unique post-translational processing is related to its interaction with the neuroendocrine protein 7B2 (Braks and Martens, 1994; Martens et al., 1994; Benjannet et al., 1995). Whereas PC2 is no longer processed after cleavage of its proregion, PC1 and PC5A are truncated at their C-terminus at the level of the TGN or in immature secretory granules (Vindrola and Lindberg, 1992; Lindberg, 1994; Zhou and Mains, 1994; De Bie et al., 1996). The C-terminal cleavage of PC1 was demonstrated to increase specific activity while decreasing overall stability of the enzyme. In addition, C-terminal cleavage of PC1 is important to narrow down the pH optimum, increase the calcium-dependence, and alter the susceptibility to certain proteinase inhibitors (Zhou and Lindberg, 1994). Indeed, a PC1 mutant in which the carboxyl-terminal segment was truncated and expressed in neuroendocrine PC12 cells was found to process the NT/NN precursor as efficiently as the wild type enzyme. Conversely, a PC1 mutant, which could not be cleaved at its C-terminal segment, processed pro-NT/NN much less efficiently (Zhou et al., 1995b).

1.2.5 Cleavage specificity of the prohormone convertases: a tightly regulated process

In order to determine potential target substrates for the prohormone convertases, their cleavage specificities were investigated *in vitro* using either cell recombinant systems co-expressing a PC and its potential target, or simply by co-incubating purified PCs with potential substrates. Some of these studies have been complemented by inhibiting PC expression *in vitro* using antisense oligonucleotides. Each PC possesses the ability to cleave numerous precursor molecules, albeit with distinct cleavage specificities. The processing of POMC provides a good example of this specificity.

When co-expressed with POMC in AtT-20 cells, PC1 preferentially processes this precursor into ACTH, β-lipotropin and, to a lesser extent, β-endorphin, whereas PC2 preferentially processes it into β-endorphin and α-melanocyte stimulating hormone (Benjannet et al., 1991; Bloomquist et al., 1991; Marcinkiewicz et al., 1993; Zhou et al., 1993; Friedman et al., 1994). Specific patterns of cleavage by either PC1 or PC2 have also been demonstrated for pro-glucagon (Rouille et al., 1994; Dhanvantari et al., 1996), pro-NT/NN (Rovere et al., 1996a), pro-thyrotropin (Schaner et al., 1997), pro-enkephalin (Breslin et al., 1993; Johanning et al., 1996; Johanning et al., 1998), pro-cholecystokinin (Yoon and Beinfeld, 1997; Wang et al., 1998), pro-somatostatin (Brakch et al., 1995) and pro-dynorphin (Day et al., 1998). Overall, both PC1 and PC2 process peptides routed to the regulated secretory pathway and appear to be specific to this route. This might also be the case for PC5A, although it still remains to be demonstrated (for review see Seidah, et al., 1999; Seidah and Chrétien, 1999; Bergeron et al., 2000).

The enzymatic activity of the PCs is tightly controlled by various factors such as local concentrations of Ca^{2+}, pH (Benjannet et al., 1992; Shennan et al., 1995; Lamango et al., 1999), the degree of maturation of the PC (pro vs mature forms) and its level of expression. In addition, two modulators of the biological activity of prohormone convertases PC1 and PC2 have been identified. These are Pro-SAAS and 7B2, respectively. 7B2 is a PC2-specific binding protein (Martens et al., 1994) which appears to be important for the proper maturation of pro-PC2; non-endocrine cells do not produce mature PC2 unless they are transfected with 7B2 (Zhu and Lindberg, 1995). This is also the case for the human neuroepithelioma SK-N-MCIXC cells which express immature forms of PC2, unless they are transfected with 7B2 (Seidel et al., 1998). Invalidation of

7B2 with an antisense oligonucleotide in endocrine cells or in an animal knockout (KO) results in the loss of PC2 enzymatic activity (Barbero and Kitabgi, 1999; Westphal et al., 1999).

Pro-SAAS has recently been identified (Fricker et al., 2000) and shown to be a potent and specific inhibitor of PC1 (Cameron et al., 2000; Fricker et al., 2000; Qian et al., 2000). Although there is no amino acid sequence homology between pro-SAAS and 7B2, these two proteins are similar in size, contain comparably high percentages of proline as well as several pairs of basic amino acids, and are both widely distributed in neuroendocrine tissues (Marcinkiewicz et al., 1986; Seidel et al., 1998; Fricker et al., 2000). Also, the inhibitory domains of both proteins are located near their C-terminus and involve pairs of basic residues (Lys-Lys in the case of 7B2, and Lys-Arg in the case of pro-SAAS). Cleavage at these pairs of basic residues eliminates their inhibitory activity. However, whereas 7B2 is required for the expression of PC2 enzymatic activity (Zhu and Lindberg, 1995), this is not the case for pro-SAAS since enzymatically active PC1 is detected in expression systems lacking pro-SAAS (Benjannet et al., 1991; Boudreault et al., 1998).

There is increasing evidence to suggest that modulation by factors such as 7B2 and pro-SAAS might play a critical role in determining the biological activity of the PCs. For instance, prolonged exposition of pancreatic β-cell-derived MIN-6 cells to free fatty-acids leads to a decreased pro-insulin to insulin conversion. Exposed cells expressed normal levels of PC1 and PC2 mRNA, but secreted higher amounts of pro-PC2 and pro-PC1 than controls, presumably through intracellular biochemical changes affecting the activation and function of the convertases (Furukawa et al., 1999). It is postulated that prolonged

exposure of β-cells to free fatty-acids may affect the biosynthesis and posttranslational processing of pro-insulin, PC2, PC1 and 7B2, and thereby contribute to the hyperproinsulinemia of type 2 diabetes (Furukawa et al., 1999). Similarly, in the animal model of insulin-induced hypoglycemic shock, where adrenomedullary 7B2 expression is decreased, the ratio of pro-PC2 to mature PC2 was increased (Seidel et al., 1998).

1.2.6 Expression of prohormone convertases in the brain

With the exception of PC4, all PCs discovered so far were found to be expressed in mammalian brain using *in situ* hybridization and/or Northern blot (Schafer et al., 1993; Dong et al., 1995; Seidah et al., 1996b; Seidah et al., 1999b). Whereas furin, PACE4 and PC7 are expressed in both neuronal and non-neuronal cells (Schafer et al., 1993; Dong et al., 1995), PC1 and PC2 are exclusively expressed in neurons (Smeekens and Steiner, 1990; Schafer et al., 1993; Dong et al., 1995; Dong et al., 1997). *In situ* hybridization experiments have suggested that this is also the case for PC5A (Dong et al., 1995).

1.2.7 PC-inactivated animals: a useful tool to study the physiological roles of the convertases

To date, the physiological role and biochemical properties of prohormone convertases have been largely inferred from *in vitro* studies. However, work by different groups or the use of different *in vitro* systems have often yielded different or even conflicting results. For instance, PC12 cells stably-transfected with pro-melanin-concentrating hormone (pro-MCH) and PC2 could fully release NEI and MCH from the precursor, whereas GH4C1 cells co-infected with PC2 and pro-MCH could not (Viale et

al., 1999). In addition, *in vitro* work can only be of predictable value provided that the convertases are co-expressed with their putative *in vivo* substrate. The recent development of transgenic mouse models inactivated for one of the convertase represents a powerful tool to study the physiological involvement of any given PC in the processing of a specific substrate (for review see Canaff et al., 1999).

The first mouse model inactivated genetically for a convertase was created by the team of Donald Steiner (Furuta et al., 1997). These authors generated a strain of mice lacking active PC2 by introducing a neomycin resistance gene (NeoR) into the third exon of the mouse PC2 gene. This gene insertion results in the synthesis of an enzymatically inactive form of PC2. PC2 null mice appear to be normal at birth and manifest a slight decrease in postnatal growth thereafter. They have chronic fasting hypoglycemia and a reduced rise in blood glucose levels following an intraperitoneal glucose tolerance test. The islets in PC2 null mice at 3 months of age show marked hyperplasia of α- and δ-cells and a relative diminution of β-cells. Consistent with the aberrant glucose metabolism and the histological abnormalities, the mice show deficient processing of pro-glucagon, pro-insulin and pro-somatostatin in α-, β- and δ-cells, respectively (Furuta et al., 1997; Furuta et al., 1998). These mutant mice have since been investigated for their ability to process a number of other neuropeptide precursors and were found defective in their processing of pro-enkephalin (Johanning et al., 1998), pro-melanin-concentrating hormone (Viale et al., 1999), pro-dynorphin (Berman et al., 2000), pro-orphanin FQ/nociceptin, POMC (Allen et al., 2001), the putative PC1 inhibitor pro-peptide pro-SAAS (Sayah et al., 2001) and pro-somatostatin (Yasothornsrikul et al., 2001), thus supporting the view that PC2 plays a key role in the endoproteolytic maturation of numerous peptide precursors.

Interestingly, these defects in the conversion of peptide precursors are specific to certain cleavage sites. Thus, PC2 null mice show a pro-dynorphin processing profile characterized by a complete lack of Dyn A-8 and a substantial reduction in the levels of Dyn A-1 and Dyn B-13 as compared to wild-type (Berman et al., 2000). In the case of POMC, PC2 null animals exhibit an increase in β-LPH and β-endorphin and a decrease in γ-LPH (Allen et al., 2001). Overall, these results demonstrate that PC2 plays an essential and specific role in the physiological conversion of numerous peptide precursors, but does not account by itself for the full maturation of propeptides.

The invalidation of 7B2 also produced interesting results (Westphal et al., 1999). Predictably, 7B2 null mice show no demonstrable PC2 activity, are deficient in their processing of islet hormones and display hypoglycemia, hyperproinsulinemia, hypoglucagonemia and decreased enkephalin levels. However, in addition to these defects similar to those found in PC2 null mice, 7B2 null mice exhibit a marked Cushing's syndrome characterized by severe bruising due to thinning of the skin and dermal atrophy, accompanied by hyperkeratosis (Westphal et al., 1999). Ninety percent of these mice do not survive to weaning and they usually die before 9 weeks of severe Cushing's syndrome secondary to ACTH hypersecretion. In the anterior lobe, the site of normal ACTH production, cleavage of POMC into full-length ACTH, is unaffected by the deletion of either 7B2 or PC2, presumably because this cleavage occurs primarily through the action of PC1 rather than PC2. Instead, ACTH accumulation seen in 7B2 null mice appears to arise from deregulated secretion from the intermediate lobe of the pituitary (Westphal et al., 1999). The reason why the deletion of 7B2 as opposed to that of PC2 should result in abnormal ACTH secretion is unclear but may be related to

additional functional roles for 7B2 not related to PC2-mediated effects. Since 7B2 is a member of the granin family (Huttner et al., 1991), it is possible that it acts by itself as a biologically active peptide able to mediate autocrine and paracrine actions, as it was shown to be the case for other granins (Tatemoto et al., 1986; Westphal et al., 1999). The hypothesis of additional roles for 7B2 is supported by the demonstration of its expression in brain areas lacking PC2 (Seidel et al., 1998).

The selective expression of PC4 in the ovary and spermatocytes/spermatids of the testis (Nakayama et al., 1992; Seidah et al., 1992a; Torii et al., 1993) suggests that this convertase is involved in reproduction functions. Accordingly, male PC4 null mice have severely impaired fertility and eggs fertilized by their spermatozoa fail to grow to the blastocyst stage (Mbikay et al., 1997). Spermatocytes from PC4 mutant animals do not exhibit any evident spermatogenic abnormality, although their ability for capacitation is severely impaired (Mbikay et al., 1997). Abnormalities were also noted in PC4-transgenic female mice, which exhibited delayed folliculogenesis in the ovaries (Li et al., 2000a; Li et al., 2000b). The molecular basis for the fertility impairment seen in PC4-deficient animals remains to be clarified but may involve the aberrant processing of the PACAP precursor reported in gonads of mutant animals (Li et al., 2000a; Li et al., 2000b).

The invalidation of the furin gene leads to a lethal phenotype since mutant embryos die between 10.5 and 11.5 days post coitus, probably of hemodynamic insufficiency associated with severe ventral closure defects and failed cardiogenesis (Roebroek et al., 1998). These results identify furin as an important activator of signals responsible for ventral closure and embryonic development.

The search for obese and diabetic mouse models led to the discovery of a mouse strain with a recessive mutation on the *fat* locus located on chromosome 8. This defect was found to result from a single point mutation at the *CPE* gene encoding the exopeptidase carboxypeptidase E (Naggert et al., 1995). The CPE activity in $CPE^{fat/fat}$ is virtually absent and mutated mice develop late onset obesity. The loss of exopeptidase function is accompanied by aberrant processing of a number of peptide precursors. In the periphery, $CPE^{fat/fat}$ mice exhibit increased levels of pro-insulin. In the brain, they exhibit markedly reduced levels (>80%) of mature NT and NN but an increase in NT-KR and NN-KR peptides, resulting from deficient removal of the C-terminal pair of basic residues residues (Rovere et al., 1996b). The production of melanin-concentrating hormone (MCH) is increased two- to three- fold in $CPE^{fat/fat}$, but there is a tenfold decrease in neuropeptide-Glu-Ile (NEI), another pro-MCH-derived product. In the pituitary, the processing of POMC is also severely impaired since POMC levels are increased to about 20 times higher than normal (Fricker et al., 1996) and the final ACTH products are found in only about 30% of the levels of those observed in the pituitary of WT mice. However, the ACTH formed is extended with basic residues at its C-terminus (Fricker et al., 1996), consistent with the absence of CPE activity. The consequence of a defect in CPE activity for the biological activity of neuropeptides will vary according to their pharmacology and location in the precursor. Peptides that are internal to the precursor and for which the integrity of the C-terminal portion of the precursor is required will be produced in inactive form (e.g. NT and NN). Peptides that are internal to the precursor and for which the integrity of the C-terminal portion is not required will be produced in active form (e.g. ACTH). Finally, neuropeptides that are located at the C-

terminus of their precursor, such as MCH will be produced normally.

The defects observed in CPE$^{fat/fat}$ mice do not seem to be restricted to the deficient removal of the C-terminal lysine and arginine residues exposed by the action of the PCs on the peptide precursor. For instance, it is unclear why elevated amounts of POMC and pro-insulin are seen in mutant animals, but this suggests that the deletion of CPE activity affects the endoproteolytic step as well, prior to removal of the pair of basic residues residues. Some evidence suggests that this is indeed the case. Day et al. have shown that PC2 cleavage of pro-dynorphin is enhanced when carried out in the presence of CPE and, conversely, blocked when CPE activity is inhibited with GEMSA, a specific CPE inhibitor (Day et al., 1998). It was also recently demonstrated that the levels of the active forms of PC1 and PC2 were differently altered in a number of brain regions as well as in the pituitary of CPE$^{fat/fat}$ mice, suggesting that some of the effects detected in these mice may in fact be secondary to alteration in the activity of the PCs (Berman et al., 2001). These results suggest that CPE may interact with the PCs. Finally, CPE has been proposed to play a role in the sorting of peptides to the regulated pathway of secretion (Cool et al., 1997; Cool and Loh, 1998; Normant and Loh, 1998), which could in itself account for some of the processing defects observed in CPE$^{fat/fat}$ mice.

1.2.8 Case studies associated with deficient prohormone conversion: Is prohormone conversion clinically relevant?

As the concept of endoproteolytic maturation becomes more pervasive, an increasing number of hormonal disorders have been associated with aberrant maturation of peptide precursors. Thus, Yano and colleagues (1992) described a Japanese female patient with

diabetes mellitus, fasting hyperproinsulinemia, and higher than normal levels of pro-insulin. The pro-insulin gene of this patient was found to bear a mutation that prevented recognition of the C-peptide-A chain pair of basic residues protease, thus resulting in an elevation of the pro-insulin-like material in the circulation. This mutation was also identified in her children. The secretion of improperly processed insulin likely accounted for the impaired glucose tolerance and postprandial hypoglycemia found in this patient. Indeed, other families have been identified in which pro-insulin mutations were associated with hyperproinsulinemia (Gabbay et al., 1979; Gruppuso et al., 1984; Burgess and Kelly, 1987; Chan et al., 1987; Carroll et al., 1988; Barbetti et al., 1990).

Another disease involving abnormal pro-insulin biosynthesis was described by O'Rahilly et al (1995). These authors described a woman with early-onset obesity, impaired glucose tolerance and mild reactive hypoglycemia, elevated plasma pro-insulin, but reduced insulin levels (O'Rahilly et al., 1995). Features of hypogonadism, hypocortisolism and elevated POMC levels were also found, suggesting the possibility of a generalized defect related to impaired processing of prohormones (O'Rahilly et al., 1995). This individual was later identified as compound heterozygous for mutations in PC1 (Jackson et al., 1997). On one PC1 gene allele, a missense replacement of an Arg for a Gly resulting from a mutation at codon 483 prevented maturation of pro-PC1, thereby causing its retention in the ER. On the other allele, a substitution of a C for an A at position +4 of the intron 5 donor splice site caused skipping of exon 5 with loss of 26 amino acids, a frameshift, and the introduction of a premature stop codon within the catalytic domain. Therefore, this patient is unable to produce functional pro-PC1. This defect most likely explains the impaired processing of POMC and secondary

hypocortisolism. Her hypogonadotropic hypogonadism may arise from impaired processing of hypothalamic hormones and neuropeptides related to gonadotropin-releasing hormone secretion. The obesity phenotype probably results from aberrant processing of neuropeptides that normally regulate appetite and calorific intake (O'Rahilly et al., 1995).

A much more subtle, region-selective defect in propeptide conversion was noted in patients suffering from Worfran syndrome (DIDMOAD; diabetes insipidus/mellitus, optic atrophy, deafness). In patients with diabetes insipidus, no mature immunoreactive vasopressin in the supraoptic nucleus was detected, although the vasopressin precursor was present. Interestingly, these patients lacked immunoreactive PC2 and 7B2 in both the supraoptic and paraventricular nuclei of the hypothalamus, but not in surrounding nuclei (Gabreels et al., 1998b). A very similar observation was noted in vasopressin neurons of two Prader-Willi syndrome patients. These results strongly suggest that the diabetes insipidus disorder of these patients arises from deficient pro-vasopressin processing due to the lack of PC2/7B2 (Gabreels et al., 1998a). The fact that, in these patients, both PC2 and 7B2 were expressed normally in surrounding tissues but were absent in the supraoptic and paraventricular nuclei of the hypothalamus, suggests that the primary defect was not due to a gene defect but rather to a more subtle, region-specific deregulations in PC2 and/or 7B2 expression. It is tempting to speculate that such defects could account for other endocrine syndromes. The implication of the PCs in an increasing number of endocrine disorders confirm that these enzymes play an important physiological role and that their deregulation can be associated with pathological states.

1.3 STATEMENT OF THE PROBLEM

Earlier studies have demonstrated that the NT/NN precursor is processed in the brain to generate the biologically active peptides NT, NN and, to a lesser extent, large NN (for review see Kitabgi et al., 1992). *In vitro* experiments performed on stably-transfected endocrine cells expressing pro-NT/NN and either PC1, PC2 or PC5A have shown that all three convertases efficiently processed pro-NT/NN, albeit each with a different maturation profile (Fig. 6). The major maturation products obtained with PC1 were NT and large NN, as well as smaller amounts of NN (Rovere et al., 1996a). PC2 generated equivalent amounts of NT and NN with very little of the large forms (Rovere et al., 1996a), whereas PC5A generated NT, large NT and large NN in equivalent amounts, but virtually no NN (Barbero et al., 1998). The patterns generated by PC1 and PC5A correspond to those observed in the gut (Carraway and Mitra, 1990; Shaw et al., 1990; Carraway and Mitra, 1991; Carraway et al., 1992) and adrenal (Carraway et al., 1992; Carraway et al., 1993), respectively. With PC2, the major products detected were NT and NN (Rovere et al., 1996a), a pro-NT/NN maturation profile similar to that found in rat brain (Kitabgi et al., 1991; de Nadai et al., 1994), consistent with the reported expression of PC2 in a number of NT-rich brain areas. PC2 therefore appears to be a good candidate for pro-NT/NN processing in the brain. However, whether PC2 plays a role in the physiological processing of pro-NT/NN remains to be determined.

PC1 is distributed extensively in the brain where it is expressed exclusively by neurons (Schafer et al., 1993; Dong et al., 1997; Winsky-Sommerer et al., 2000) and is therefore another potential candidate. In the case of PC5A, it is less clear whether it may

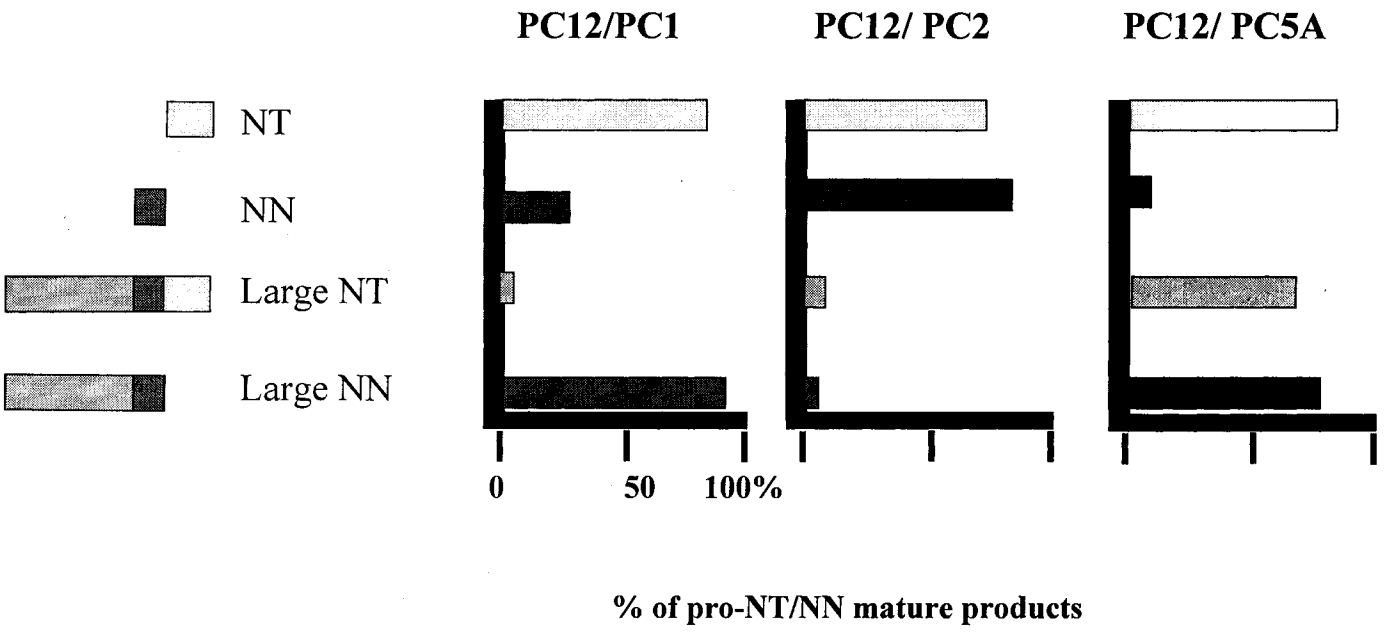

Figure 6: ***Differential processing of pro-NT/NN in neuroendocrine PC12 cells transfected with either PC1, PC2 or PC5A.*** Note that each prohormone convertase exhibits a specific maturation profile. Whereas the maturation obtained when PC12 cells are transfected with PC2 is reminiscent to the maturation profile observed in the brain, the maturation products of PC1- and PC5A-transfected cells are reminiscent to what is seen in the gut and brain, respectively.

play a role in pro-NT/NN processing since, although it has been shown to be expressed in the brain (Dong et al., 1995; Dong et al., 1997), it is not known whether it is selectively expressed by neurons and, if so, whether its distribution is compatible with a role in pro-NT/NN processing.

1.4 AIM OF THE STUDY

The convertase PC5A has only recently been cloned and mounting evidence suggests that it may be implicated in the processing of peptide precursors routed to the regulated secretory pathway. In order to gain further insight into the physiological role of this convertase in the brain, immunohistochemistry was employed to describe the regional, cellular and subcellular distribution of this enzyme in rat brain. These results are presented in Chapter 2.

The remaining portion of my work focuses on the elucidation of the mechanisms involved in the processing of the precursor of NT in mammalian brain, *in vivo*. Pro-NT/NN is an ideal model for the study of propeptide maturation in the brain since: (1) its distribution has been consistently described to be restricted to discrete nuclei throughout the brain; (2) it is a relatively simple precursor, which upon maturation yields at least four well characterized biologically active peptides, namely NT, NN, large NT and large NN; (3) site-specific antibodies have been developed that recognize its maturation products, thus allowing for their radioimmunological and immunohistochemical detection.

In a series of experiments presented in Chapters 3 and 4, we have used anatomical tools to investigate evidence in support of a physiological role for PC1, PC2 and PC5A in the maturation of pro-NT/NN in the brain. Specifically, the immunohistochemical

distribution of NT was compared to that of PC1, PC2 and PC5A, first in serial rat brain sections, and then in dually stained sections to determine the extent of cellular co-localization of NT with either protease. Also, the subcellular localization of PC1, PC2, PC5A, NT and another maturation product of pro-NT/NN, the exposed Glu^{134}-Ile^{139} (E6I; Fig. 2) sequence of pro-NT/NN, were examined in relation to that of subcellular compartments markers to gain insight into the intracellular sequence of pro-NT/NN processing.

In Chapter 5, we have investigated the maturation profile of pro-NT/NN in the brain of mice inactivated for PC7 (PC7-/-), PC2 (PC2-/-), and/or PC1 (PC1+/- and PC2-/-; PC1+/-) in order to gain further insight into the role of these convertases in the processing of pro-NT/NN, *in vivo*. Pro-NT/NN maturation products were quantified by a combination of radioimmunoassay and high pressure liquid chromatography techniques (HPLC). Comparison of the pro-NT/NN maturation profiles between wild type and KO mice will allow us to determine the relative contribution of PC1, PC2 and PC5A in the maturation of pro-NT/NN in the rat brain. Overall, these studies should provide us with a better understanding of the functional role of the convertases in the central processing of the NT/NN precursor.

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Chapter Two

Immunohistochemical distribution of the prohormone convertase PC5A in rat brain

IMMUNOHISTOCHEMICAL DISTRIBUTION OF THE PROHORMONE CONVERTASE PC5A IN RAT BRAIN

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Abbreviations used: PC, prohormone convertase; TBS, Tris buffered saline; FITC, fluorescein isothiocyanate; NGS, normal goat serum; ABC, avidin-biotin complex

ABSTRACT

Prohormone convertase 5 (PC5) is an endoprotease of the kexin/subtilisin-like family which has been postulated to play a role in the proteolytic maturation of a variety of propeptides in mammalian brain. In order to gain insight into the functional role of PC5 in the central nervous system, the regional, cellular and subcellular distribution of the enzyme was investigated by immunohistochemistry in rat brain using a N-terminal-directed specific antibody previously shown to recognize both mature and unprocessed forms of the enzyme. Throughout the brain, PC5A-immunoreactivity was concentrated within nerve cell bodies and proximal dendrites. No PC5A immunoreactivity was associated with astrocytes, as confirmed by the absence of PC5A immunolabeling in cells immunopositive for the glial protein S-100α. Within neurons, PC5A immunoreactivity was concentrated within the Golgi apparatus, as revealed immunohistochemically within the same sections using antibodies against the medial cisternae protein MG-160. It was also present within small vesicular-like elements distributed throughout the cytoplasm of perikarya and dendrites, but not of axons as confirmed by its lack of co-localization with the synaptic terminal marker α-dynamin. These results suggest that PC5A is active within early compartments the neuronal regulated secretory pathway and that it is unlikely to be released with its processed substrates. At the regional level, PC5A-immunoreactive perikarya were extensively distributed throughout the forebrain. The most numerous and intensely labeled cells were detected in the olfactory bulb, cerebral cortex, globus pallidus, endopedoncular and subthalamic nuclei, septum, diagonal band of Broca, magnocellular and medial preoptic areas, supraoptic and arcuate nuclei of the hypothalamus, and anterodorsal, laterodorsal, paraventicular and reticular nuclei of the thalamus. Moderate to dense neuronal labeling was also evident in the olfactory tubercle, caudoputamen, claustrum, bed nucleus of the stria terminalis, substantia innominata, hippocampus, amygdala, and remaining thalamic and hypothalamic nuclei. This

widespread distribution suggests that PC5A is involved in the processing of a variety of neuropeptide and/or neurotrophin precursors in mammalian brain.

Key words: Prohormone convertase, PC5, neuropeptides, immunohistochemistry, peptide processing, kexin.

INTRODUCTION

Neuropeptides are synthesized as large molecular precursors that are subsequently cleaved, usually at the level of dibasic residues, to release biologically active peptide fragments. The first enzyme recognized as being responsible for the proteolytic maturation of a peptide precursor was the yeast enzyme Kex2 (yeast kexin). Kex2 is a Ca^{2+}-dependent serine protease with a subtilisin/kexin-like catalytic domain which is involved in the processing of the pro-α-mating factor and pro-killer toxin in yeast (Julius et al., 1984; Fuller et al., 1989). Since then, seven mammalian prohormone convertases (PC) have been identified on the basis of their homology with Kex2. All PCs of that family are Ca^{2+}-dependent serine proteases with a highly conserved catalytic domain. These include PC1 (Seidah et al., 1990; Seidah et al., 1991) (also called PC3; Smeekens et al., 1991)), PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990), furin (Roebroek et al., 1986), PACE4 (Kiefer et al., 1991), PC4 (Nakayama et al., 1992; Seidah et al., 1992), PC5 (Lusson et al., 1993; Nakagawa et al., 1993) and PC7 (Seidah et al., 1996b), also referred to as PC8 (Bruzzaniti et al., 1996) or LPC (Meerabux et al., 1996).

With the exception of PC4, all PCs discovered so far were found to be expressed in the mammalian brain using *in situ* hybridization and/or Northern blot (Kiefer et al., 1991; Schafer et al., 1993; Dong et al., 1995; Seidah et al., 1996b). While furin, PACE4 and PC7 are expressed in both neuronal and non-neuronal cells (Schafer et al., 1993; Dong et al., 1995; Seidah et al., 1996b), PC1, PC2 and PC5 appear to be primarily associated with neurons (Smeekens and Steiner, 1990; Schafer et al., 1993; Dong et al., 1995). There is still little information available on the function of these prohormone convertases in the central nervous system (CNS), although several have been proposed to play a role in the processing of neuropeptides (Schafer et al., 1993; Dong et al., 1995; Dong et al., 1997) and neurotrophins (Seidah et al., 1996a; Seidah et al., 1996c). Recent evidence has

accumulated to suggest that PC5 (also designated PC6; Nakagawa, et al., 1993), may be particularly active in this regard.

PC5 exists as two isoforms, PC5A and PC5B, resulting from the alternative splicing of a common precursor (Nakagawa et al., 1993). These isoforms differ only at their C-terminus, PC5B being a C-terminal extended isoform of PC5A. Prepro-PC5, as all known PC proenzymes, possesses a signal peptide, a prosegment, a highly conserved catalytic domain, a segment termed P-domain thought to be critical for the catalytic activity of the enzyme, and an enzyme-specific C-terminal segment. While PC5A exists as a soluble protein, PC5B is a type I membrane-associated protein exhibiting both a C-terminal hydrophobic transmembrane sequence and a cytosolic tail.

To date, PC5A and/or PC5B have been demonstrated *in vitro* to possess the ability to process the neuropeptide pro-neurotensin (Barbero et al., 1998), the prohormone pro-renin (Nakagawa et al., 1993; Laframboise et al., 1997), the precursor of the von Willebrand factor (Creemers et al., 1993), the constitutively secreted viral surface glycoproteins haemagglutinin of virulent avian influenza viruses (Horimoto et al., 1994), the bovine leukemia virus enveloppe glycoprotein gp73 (Zarkik et al., 1997), the Müllerian inhibiting factor (Nachtigal and Ingraham, 1996), the membrane-associated receptor protein tyrosine phosphatase (Campan et al., 1996), and the neurotrophins nerve growth factor (Seidah et al., 1996c), neurotrophin-3, and brain-derived neurotrophic factor (Seidah et al., 1996a).

In situ hybridization studies have previously documented a widespread expression of PC5A in rat brain (Dong et al., 1995). Nothing is known, however, of the distribution of the protein itself at either regional, cellular or sub-cellular levels. Northern blot analyses have shown that the major form of PC5A mRNA present in rat brain corresponds to the 3.8 kb PC5A isoform of the enzyme (Nakagawa et al., 1993; Dong et al., 1995). Furthermore, this isoform appears to mainly exist as a 65 kDa C-terminal-truncated

processed form, at least according to measurements made in mediobasal hypothalamic extracts (Dong et al., 1997). This is in contrast to what was observed in transfected neuro-endocrine cells, in which both 117 kDa and 65 kDa mature forms of the peptide were shown to co-exist (De Bie et al., 1996; Barbero et al., 1998). In the present study, we examined the regional, cellular and subcellular distribution of the processed PC5A protein in the rat forebrain using immunohistochemistry, in an attempt to gain insight into the functional role of this convertase in the CNS.

EXPERIMENTAL PROCEDURES

Antibodies

The PC5A antiserum was obtained from rabbits immunized against an octopus branched synthetic peptide as described previously (Posnett et al., 1988). The peptide used contained amino acids 116-132 from the following N-terminal segment of mouse PC5A: Asp-Tyr-Asp-Leu-Ser-His-Ala-Gln-Ser-Thr-Tyr-Phe-Asn-Asp-Pro-Lys (Basak et al., 1995). The axon terminal marker was a mouse monoclonal antibody directed against human dynamin-1 (Upstate biotechnology, NY; Hinshaw and Schmid, 1995; (Warnock et al., 1995). The glial marker was a mouse monoclonal antibody directed against the α-chain of the bovine S-100 protein (Sigma Immuno Chemicals, MO; (Korr et al., 1994)). Finally, the Golgi marker was a monoclonal antibody directed against rat MG-160, a constitutive protein of the medial cisternae of the Golgi apparatus (Gonatas et al., 1995). The latter antibody was a generous gift from Nicholas K. Gonatas (University of Pennsylvania).

Tissue fixation

Adult male Sprague-Dawley rats (240-260 g) were deeply anesthetized with sodium pentobarbital (80 mg/kg i.p.) and perfused transaortically with 500 ml of a mixture of 4 % paraformaldehyde and 0.2 % picric acid in 0.1 M phosphate buffer, pH 7.4. The brain was dissected out of the skull and postfixed for 40 min by immersion in the same fixative, cryoprotected for 24 h by immersion in a 30 % sucrose solution in 0.2 M phosphate

buffer, pH 7.4 at 4°C, frozen in isopentane at -50 °C, and sectioned at a thickness of 30 μm on a freezing microtome.

Single-labeling immunohistochemistry

Brain sections were collected in phosphate buffer, pH 7.4 and processed for immunohistochemistry using a biotinylated tyramine-amplified version of the avidin-biotin peroxidase method (Adams, 1992). Briefly, after two rinses in 0.1 M Tris buffer saline, pH 7.4 (TBS), the sections were treated for 20 min with 0.3 % H_2O_2 in TBS to quench endogenous peroxidase. They were then incubated for 30 min at room temperature in NGS (3 % in TBS) and overnight at 4°C in a TBS solution containing 1 % normal goat serum (NGS), 0.2 % Triton X-100 and 1/5000 PC5A antibody. Negative control sections were incubated with PC5A antibodies pre-adsorbed with 9 X 10^{-5} M of the antigenic peptide or in the absence of primary antibodies. Following two rinses in TBS containing 1 % NGS, the sections were incubated for 50 min at room temperature with biotinylated goat anti-rabbit immunoglobulins (Jackson ImmunoResearch; 1/100) and then for 50 min with an avidin-biotin peroxidase complex (Vector ABC). The sections were then rinsed twice in TBS, transferred to a TBS solution containing 0.1 % biotinylated tyramine (NEN-Dupont) and 0.01 % H_2O_2 for 10 min, and reincubated for 50 min in the avidin-biotin peroxidase complex. Bound peroxidase was visualized by immersion of the sections in 0.01 M Tris buffer, pH 7.6, containing 0.05 % 3,3' diaminobenzidine, 0.04 % nickel chloride and 0.01 % H_2O_2. Finally, sections were mounted on gelatin-coated slides, dehydrated in graded ethanols, defatted in xylene and coverslipped for light microscopic observation.

Double-labeling immunohistochemistry

Sections prepared as above were treated for 30 min in 3 % NGS in TBS and incubated overnight at 4°C in a solution containing 0.1 M TBS, 1 % NGS, 0.2 % Triton X-100 and a

mixture of PC5A (diluted 1:200) and of either MG-160 (1:25), S-100 (1:500) or Dynamin-1 (1:100) antibodies. The following day, sections were rinsed in 0.1 M TBS containing 1 % NGS and incubated for 50 min in the same buffer containing a mixture of biotinylated goat anti-rabbit IgGs and fluorescent goat anti-mouse IgGs, both at a dilution of 1:100. They were then rinsed and incubated for 50 min in ABC solution, rinsed again, incubated as above for 10 min in a biotinyl-tyramine solution, and reincubated in ABC. After several buffer washes, sections were incubated for 50 min in a solution of Texas Red-conjugated streptavidin (1:300) (Jackson ImmunoResearch). Sections were then rinsed in TBS and mounted in Aquamount. The absence of cross-reactivity of the secondary antibodies was verified by omitting one or both primary antibodies during the overnight incubation. Double-labeled sections were analyzed by confocal microscopy using a Zeiss confocal laser scanning microscope (CLSM) equipped with a Zeiss inverted microscope and an argon/krypton laser (488 and 568 ηm). Images were processed using the Carl Zeiss CLSM software (version 3.1). Images were acquired sequentially for the two fluorofores (FITC and Texas Red) by single optical plane acquisition averaged over 32 scans per frame.

RESULTS

Cellular and subcellular localization of PC5A immunoreactivity in rat brain

Amplified peroxidase and fluorescence immunohistochemistry yielded identical distributional patterns of PC5A immunoreactivity throughout the brain. In all labeled areas, the immunoreactive enzyme was selectively concentrated within nerve cell bodies and proximal dendrites. In peroxidase-reacted material, the immunoreactivity formed small intracytoplasmic puncta scattered over the cytoplasm of both perikarya and dendrites (Fig. 1). By confocal microscopy, the labeling was found to be concentrated both within small intra-cytoplasmic granules and within a thick juxtanuclear crescent reminiscent of the Golgi apparatus. (Fig. 2A). Double labeling studies using anti-PC5A and a monoclonal antibody directed against the medial cisternae Golgi protein MG-160 (Gonatas et al., 1995) confirmed the association of PC5A immunoreactivity with the Golgi complex (Fig 2B-D). They also showed the enzyme to be present in compartments more central and peripheral to the cisternae immunoreactive for MG-160.

The overall granularity of PC5A immunoreactivity made it difficult to assess whether the enzyme was also associated with axon terminals. To settle this issue, double immunostaining for PC5A and for the axon terminal protein α-dynamin was performed (Hinshaw and Schmid, 1995; Warnock et al., 1995). As can be seen in Fig. 2E, dynamin-immunoreactive axon terminals were detected as intensely fluorescent puncta distributed throughout the neuropil. Many of these puncta were seen to surround PC5A immunoreactive cells (Fig. 2E). In no instance, however, were any of these dynamin-labeled terminals found to co-localize PC5A.

In order to determine whether PC5A immunoreactivity was associated with astrocytes, double immunolabeling for PC5A and the astrocytic protein S-100 was performed in selected brain regions. By confocal microscopy, S-100-immunoreactive cells exhibited the typical stellate appearance of protoplasmic/fibrous astrocytes and

pervaded all structures exhibiting PC5A-immunoreactivity. However, none of the S-100 immunoreactive profiles double-stained for PC5A (Fig. 2F). Finally, PC5A immunoreactivity was undetectable in sections incubated either in the absence of primary antibody (Fig. 3B) or with immune serum preabsorbed with the PC5A antigenic peptide (Fig. 7B).

Topographic distribution of PC5A-immunoreactive neurons

PC5A immunoreactive neurons were widely but selectively distributed throughout the rat forebrain. A semi-quantitative estimate of this distribution is provided in Table 1. The nomenclature is based on the atlas of Paxinos and Watson (1985).

Rhinencephalon. In the olfactory bulb, intense immunostaining was detected within the perikarya of virtually all mitral cells as well as within the dendritic extensions of these cells in the external plexiform layer (Fig. 3A). Sparsely distributed, but strongly immunoreactive perikarya and dendrites were also seen in the external plexiform layer (Fig. 3C). Very few small periglomerular cell bodies were immunoreactive in the glomerular layer. The anterior olfactory nuclei exhibited a moderate number of labeled neurons, mainly within the external portion of the nuclei (Fig. 3A).

Olfactory tubercle. A few, moderately to highly immunoreactive neurons were uniformly scattered throughout the islands of Calleja (Fig. 4B).

Cerebral cortex. Both the allocortex (orbital, insular and entorhinal) and neocortex (frontal, parietal, temporal and occipital) showed heterogeneously distributed PC5A immunoreactivity. In the orbital cortex, few immunolabeled neurons were scattered throughout layers III, IV and V. In the insular cortex, only very few, lightly labeled

neurons were detected in layer V. In the cingulate cortex, a small number of PC5A-immunoreactive perikarya were detected mainly in layers II and III, and V. Very few moderately immunoreactive neurons were scattered throughout the entorhinal cortex.

Within the frontal, parietal, temporal and occipital cortices, prominent PC5A immunolabeling was observed within pyramidal neurons in layer V, as well as within the apical dendrites of these cells in more superficial layers (Fig. 4C). PC5A immunoreactivity was also detected within stellate cells within layers II, III and VI. However, these were both less numerous and less intensely labeled than pyramidal neurons.

Basal ganglia. Very few weakly immunopositive neurons were detected in the shell and core of the nucleus accumbens. Large, intensely labeled neurons were dispersed throughout the caudate/putamen, being particularly abundant in the ventro-lateral portion of the nucleus (Fig. 4). Here again, the immunoreactivity extended into the proximal processes of these cells. In addition, numerous medium-sized neurons, less intensely immunoreactive and labeled only at the level of their cell body, were detected throughout the same region (Fig. 4). The globus pallidus exhibited numerous, intensely immunoreactive neurons throughout its rostro-caudal extent. The immunoreactive processes of these neurons were particularly long and arborized (Fig. 5). Numerous neuronal perikarya and dendrites were also present in the endopeduncular and subthalamic nuclei. Very few, moderately labeled neurons were detected in the claustrum.

Basal forebrain. In the lateral septum, numerous immunoreactive neurons were present, particularly in the caudal region. These neurons were spindle-shaped and only moderately labeled. Immunolabeled neurons were more numerous, but also moderately

reactive, in the medial and triangular septum. Few moderately labeled neurons were observed in the bed nucleus of the stria terminalis. These were more abundant and more intensely labeled in the ventral than in the dorsal portion of the nucleus. In the diagonal band of Broca and magnocellular preoptic area, most constitutive neurons exhibited intense immunoreactivity over their perikarya and dendrites. Very few, and only moderately labeled, neurons were detected in the endopiriform nucleus. Numerous densely labeled neurons were detected in the anterior portion of the substantia innominata. These were fewer in number and less intensely labeled in the posterior than in the anterior tier.

Hippocampus. A subpopulation of moderately to highly immunoreactive neurons were evident within the pyramidal cell layer of the hippocampus (Fig. 6). These cells were most numerous in the CA2 and lateral portion of the CA3 subfield. Immunopositive neurons were more scarce but equally strongly labeled in the CA1 and medial portion of the CA3 subfields than in the CA2 and lateral portion of the CA3 subfields. A few labeled cell bodies were also observed in the strata oriens and radiatum in all subfields (Fig. 6B). In the dentate gyrus, a few labeled cell bodies were detected in the granule cell layer. A higher number of labeled neurons was also evident in the hilus.

Amygdala. Within the amygdaloid nuclei, PC5A immunoreactivity was weak and confined to neurons detected only in the basolateral and central nuclei.

Epithalamus. In the rostral portion of the medial habenula, PC5A immunoreactive neurons were numerous and moderately labeled whereas in the caudal portion of the nucleus they were fewer in number and less intensely reactive. In the lateral habenula,

immunoreactive neurons were as numerous, but less intensely labeled, than in the rostral portion of the medial habenula.

Thalamus. The thalamus was the brain region which expressed the highest levels of PC5A-immunoractivity (Table 1). The anterodorsal, laterodorsal, paraventricular and reticular nuclei were the most intensely labeled of all thalamic nuclei (Figs. 7A,8). In both anterodorsal and laterodorsal nuclei, immunoreactive neurons were uniformly distributed throughout (Fig. 7A). In the paraventricular and reticular nuclei, neurons were as intensely labelled but somewhat less densely packed than in either of the former two. All of the remaining thalamic nuclei exhibited a similar pattern of immunoreactivity, with numerous but only weakly to moderately labelled neurons uniformly distributed throughout.

Hypothalamus. Large numbers of PC5A immunoreactive cells were evident throughout the hypothalamus (Table 1). Most highly labelled was the supraoptic nucleus, all neurons of which were intensely immunoreactive (Fig. 8). Numerous, moderately to densely immunolabeled neurons were also evident in the medial preoptic area, arcuate nucleus and supramammillary nuclei. Moderately immunoreactive neurons were evident in the magnocellular portion of the paraventricular nucleus and in the suprachiasmatic nucleus (Fig. 8). Finally, a few moderately to lightly labeled neurons were detected in the lateral preoptic area, periventricular nucleus, anterior hypothalamic area, lateral hypothalamus (Fig. 8), ventromedial nucleus, dorsomedial nucleus, medial tuberal nucleus, premammillary nucleus and mammillary nucleus (Table 1).

TABLE, FIGURES AND FIGURE LEGENDS

TABLE 1: REGIONAL DISTRIBUTION OF PC5 IMMUNOREACTIVITY IN THE RAT CNS

Region	Labeling intensity
Olfactory bulb	
Periglomerular cells of the glomerular layer	+
External plexiform layer	+
Mitral cell layer	++++
Anterior olfactory bulb	+/-
Anterior olfactory nuclei	
Dorsal	+/-
Lateral	+
Medial	-
Ventral	+/-
External	+
Olfactory tubercle	
Islands of Calleja	++
Cerebral cortex	
Orbital cortex	
Layers III, IV and V	+
Insular cortex	
Layer V	+/-
Cingulate cortex	
Layers II, III and V	+
Neocortex	
Layer I	-
Layer II	+/-
Layer III	+/-
Layer IV	-/-
Layer V	+++
Layer VI	+/-
Basal ganglia	
Nucleus Accumbens	+/-
Caudate/putamen	++
Globus pallidus	++++
Endopenduncular nucleus	+++
Subthalamic nucleus	+++
Claustrum	+
Basal forebrain	
Septum	
Lateral	++
Medial	+++
Bed nucleus of the stria terminalis	+
Diagonal band of Broca	++++
Magnocellular preoptic area	+++
Endopiriform nucleus	+/-
Substantia innominata	
Anterior	++
Posterior	+
Hippocampal formation	
Amon's horn (CA1)	+/-
Amon's horn(CA2)	+
Amon's horn(CA3)	+/-
Dentate gyrus	
Molecular layer	-
Granule cell layer	+
Subiculum	+
Septohippocampal nucleus	+
Amygdala	
Basolateral nucleus	+
Central nucleus	++
Epithalamus	
Medial habenular nuclei	+
Lateral habenular nuclei	+

Thalamus	
Anteroventral nucleus	++
Paraventricular nucleus	+++
Paratenial nucleus	++
Anterodorsal nucleus	+++
Anteromedial nucleus	++
Reticular nucleus	+++
Reuniens nucleus	++
Rhomboid nucleus	++
Centromedian nucleus	++
Mediodorsal nucleus	++
Ventrolateral nucleus	++
Laterodorsal nucleus	+++
Ventromedial nucleus	++
Anteromedial nucleus	++
Laterodorsal nucleus	++
Gelatinosus nucleus	+
Gustatory nucleus	+
Parafascicular nucleus	+
Centrolateral nucleus	++
Ventroposterior nucleus, medial	++
Ventroposterior nucleus, lateral	++
Posterior thalamic nuclear group	++
Lateral geniculate nucleus	++
Medial geniculate nucleus	++
Ethmoid nucleus	+
Subparafascicular nucleus	+
Lateral posterior thalamic nuclei	+/-
Posterior thalamic triangular	++
Lateral posterior nucleus	++
Precommissural nucleus	+/-
Dorsolateral nucleus	++
Posterior thalamic area	+/-
Zona incerta	+
Hypothalamus	
Medial preoptic area	+++
Lateral preoptic area	+
Periventricular nucleus	+/-
Paraventricular nucleus	++
Supraoptic nucleus	++++
Suprachiasmatic nucleus	++
Anterior hyothalamic area	+/-
Lateral hypothalamus	+/-
Lateroanterior hypothalamus	+/-
Ventromedial nucleus	+
Arcuate nucleus	+++
Dorsomedial nucleus	+
Medial tuberal nucleus	+
Premammillary nucleus	+
Mammillary nucleus	+
Supramammillary nucleus	+++

[11]Semi-quantitative analysis of the number and labeling intensity of PC5 immunoreactive cells: +/-, limit of detection; +, low; ++, moderate; +++, high; ++++, very high.

Fig. 1. PC5A immunoreactive neurons in the globus pallidus. Immunoreactive hot spots are visible within the cytoplasm of large perikarya (arrow) as well as along neuronal processes (arrowheads). Scale bar = 50 μm.

Fig. 2. Confocal microscopic images of PC5A-immunoreactive neurons in the lateral hypothalamus (A, E, F) and cerebral cortex (B-D). Double immunohistochemical labeling of PC5A (in red) and of the medial cisternae Golgi protein MG-160 (in green). A: Pseudocolor imaging of PC5A-immunoreactive neurons. Note that the immunoreactivity is concentrated in small punctate organelles (arrowheads) as well as within a perinuclear ring structure (arrow). B-D: Double immunohistochemical labeling of PC5A (in red; B) and of the medial cisternae Golgi protein MG-160 (in green; C). As apparent in the fusion of images B and C (in D), PC5A immunoreactivity is concentrated in the compartment immunopositive for MG-160 (in yellow), but also extends more centrally and distally than the medial cisternae marker. E: Dual immunohistochemical labeling of PC5A (in red) and of the axon terminal protein, dynamin (in green). Fused images. Note the punctate appearance of dynamin immunoreactivity (e.g. arrows) against the cellular staining of PC5A. A few dynamin-immnoreactive terminals are seen abbutting PC5A-immunoreactive cells (arrowheads), but none are immunopositive for PC5A. F: Double immunohistochemical labeling for PC5A (in red) and the glial protein S-100α (in green). Fused images. S-100α-immunoreactive astrocytes are detected amongst, but never together with, PC5A-immunoreactive cells. Scale bar = 15 μm.

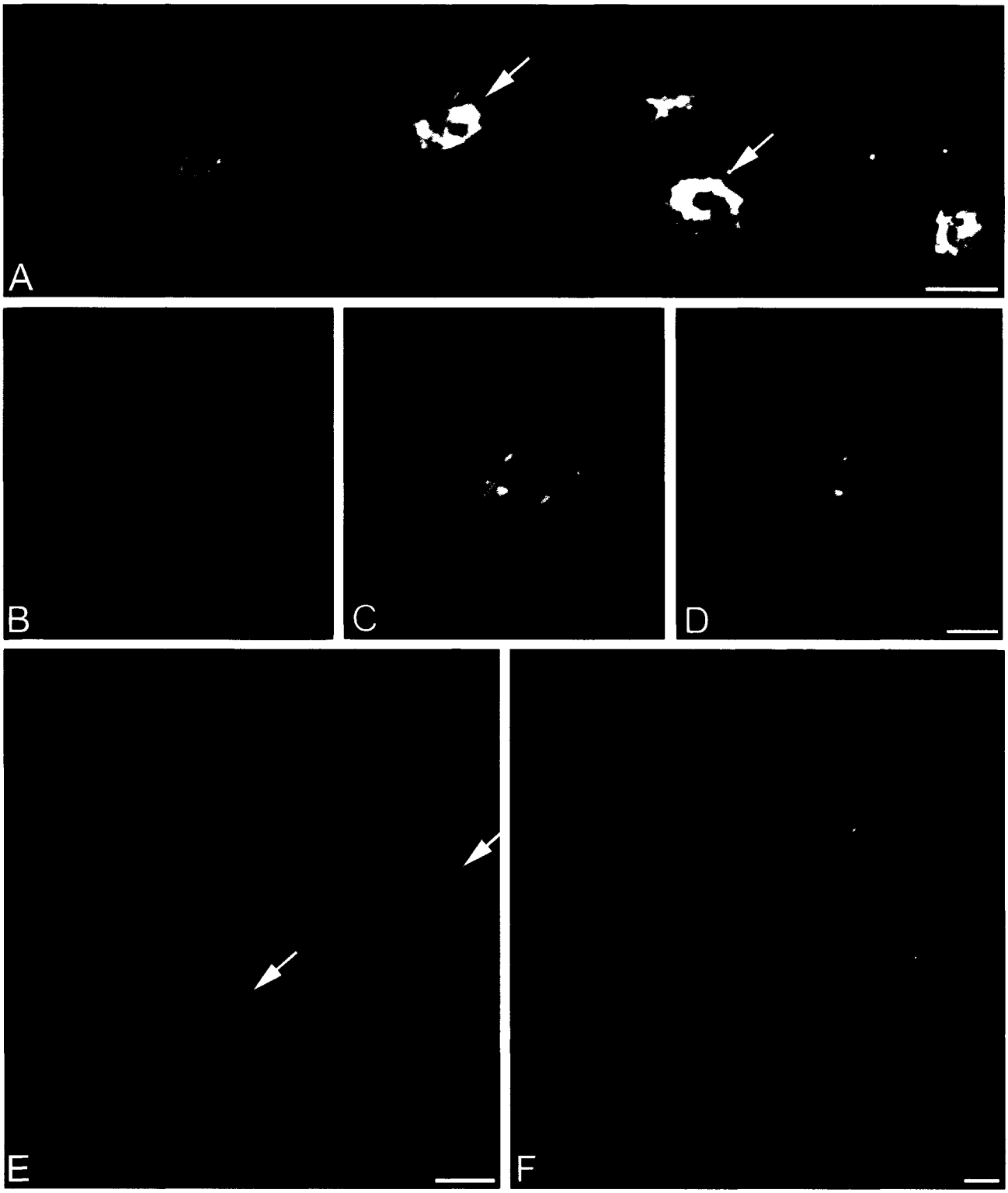
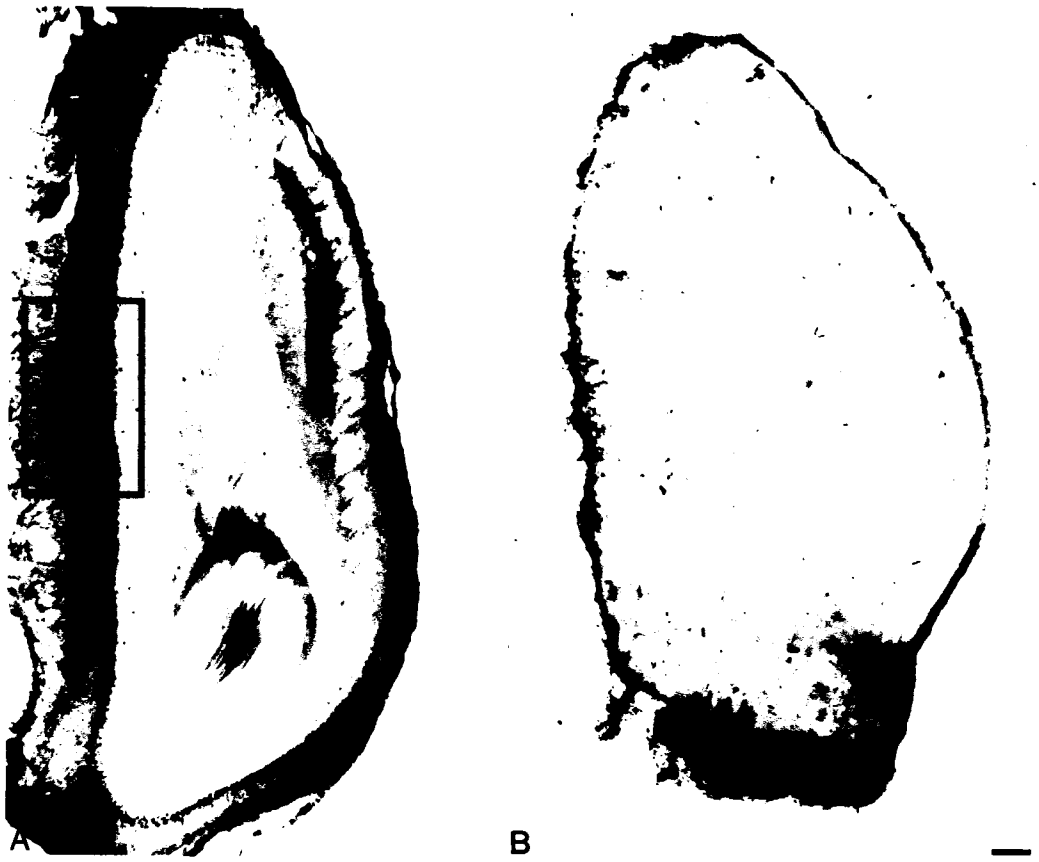

A
B
C
D
E
F

Fig. 3. A: Light microscopic distribution of PC5A immunoreactivity in rat olfactory bulb. B: Control section in which the primary antibody was omitted. C: High magnification of framed area in A. Immunolabeling is prominent in neurons of the mitral cell layer (Mtr), the dendrites of which extend into the external plexiform layer (epl). Note the sparsely distributed, but strongly immunoreactive neurons in the external plexiform layer (arrows). Note also the moderately immunoreactive periglomerular cells scattered in the glomerular cell layer (GL;arrowheads). Scale bar = 250 μm.


A
B

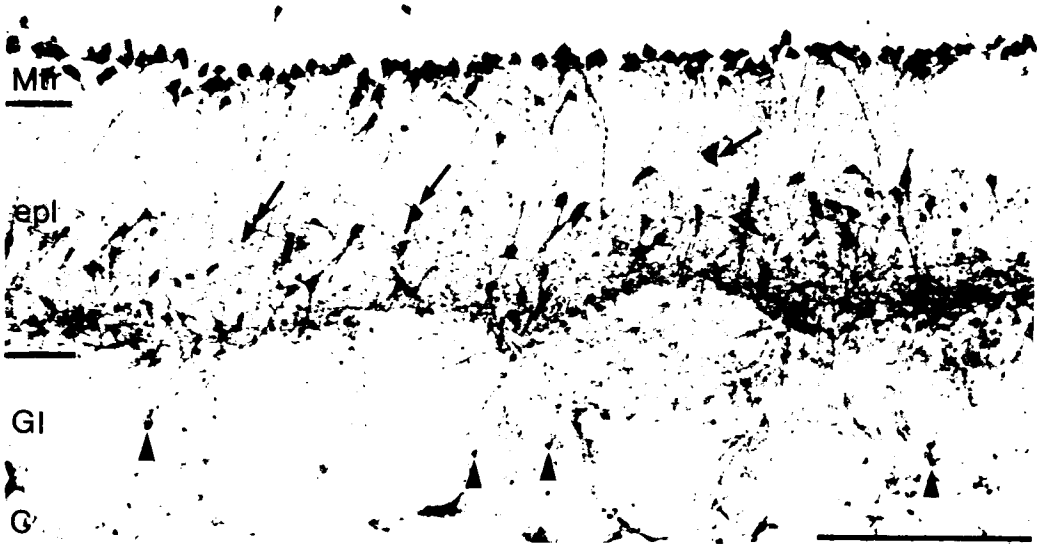
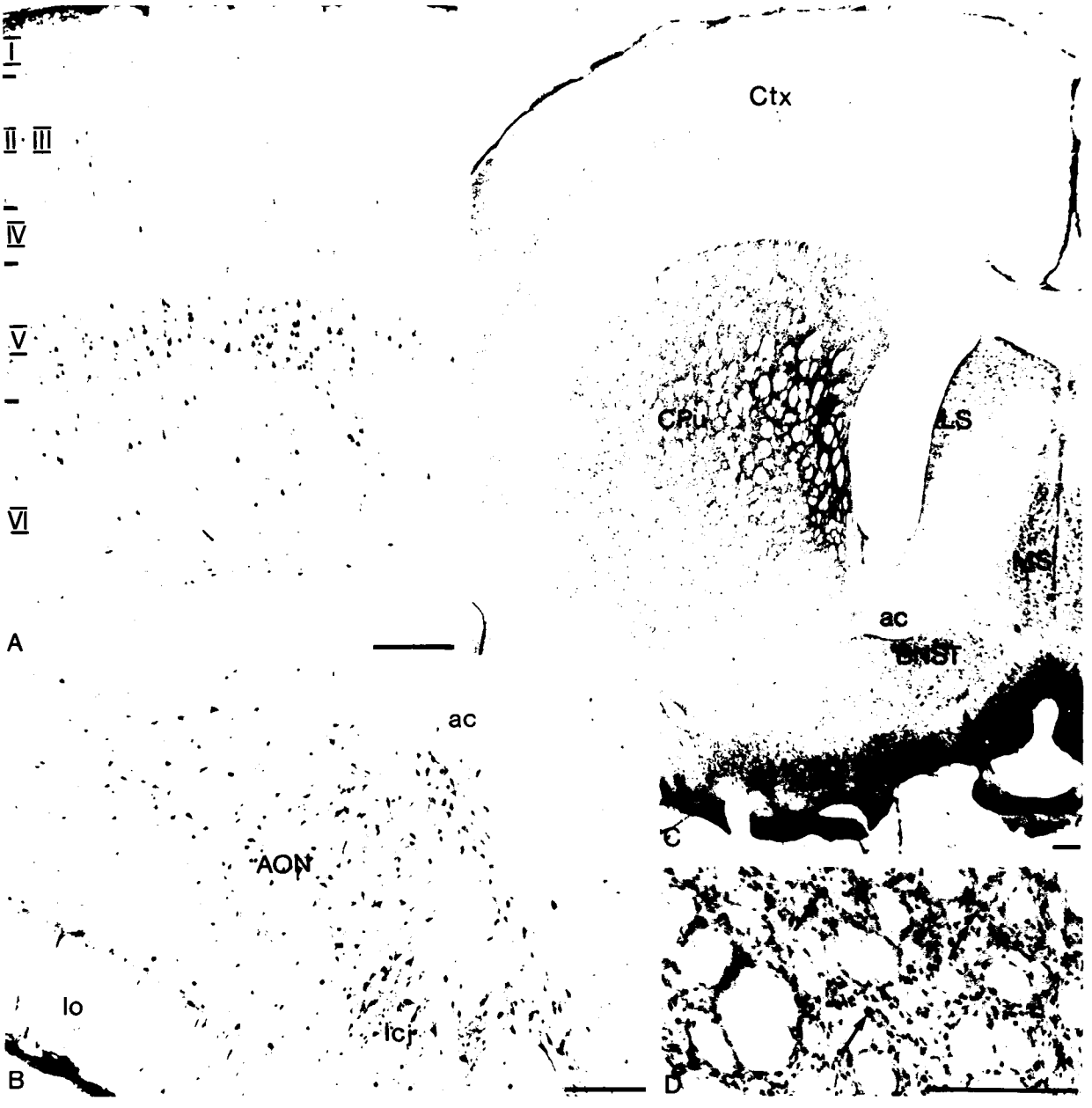

Mtr
epl
Gl
G

Fig. 4. Distribution of PC5A immunoreactivity in the frontal cortex (A), olfactory tubercle (B), rostral forebrain (C) and caudate/putamen (D). A: Frontal cortex: PC5A immunoreactivity predominates over neurons and their apical dendrites in layer V. Sparse labeled neurons are also evident in layer II-IV and VI. B: Olfactory tubercle: immunoreactive neurons are apparent in the islands of Calleja (Icj) and posterior anterior olfactory nuclei (AON); *Lo*, lateral olfactory tract; *ac*, anterior commissure. C: Rostral forebrain. Strong immunolabeling is apparent in cortical layer V (Ctx), in the dorsolateral portion of the lateral septum (LS), as well as in the medial septum (MS), medial preoptic area (MPA), ventral portion of the bed nucleus of the stria terminalis (BNST), magnocellular preoptic area (MCPO) and caudate/putamen (CPu). D: Caudate/putamen: Large, strongly immunoreactive neurons (arrows) are scattered among medium-sized, less intensely labeled ones. Scale bar = 500 μm.


I
II · III
IV
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BNST
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D

Fig. 5. Immunolocalization of PC5A in the globus pallidus. A: Low magnification. Large, strongly immunoreactive neurons are evident throughout the globus pallidus (GP) in between the myelinated fascicles of the internal capsule (ic). Note the smaller size and weaker labeling of medium-sized neurons in the adjoining caudoputamen (CPu). B: High magnification. PC5A immunoreactivity is most prominent in neuronal perikarya (arrowheads), but also extends distally in neuronal processes (arrows). Scale bar = 100 μm.

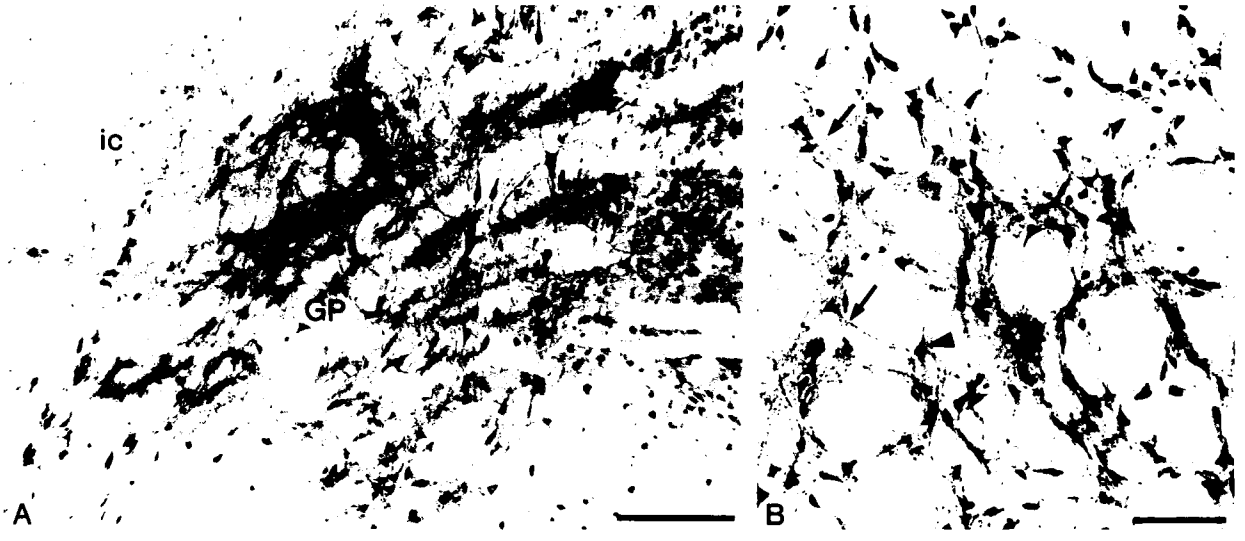

ic
GP
A
B

Fig. 6. PC5A immunoreactivity in the hippocampus. A: Low magnification. Immunolabeled neurons are scattered throughout CA1, CA2 and CA3 subfields of the hippocampus, as well as within the granule cell layer and hilus of the dendate gyrus (DG). Note the presence of immunoreactive cells in the pyramidal cell layer (pyr), as well as in the stratum oriens (or) and radiatum (ra) of all subfields. Scale bar = 500 μm. B: High magnification (CA1). A subpopulation of neurons are labeled in the pyramidal cell layer and in the stratum radiatum. Scale bar = 50 μm.

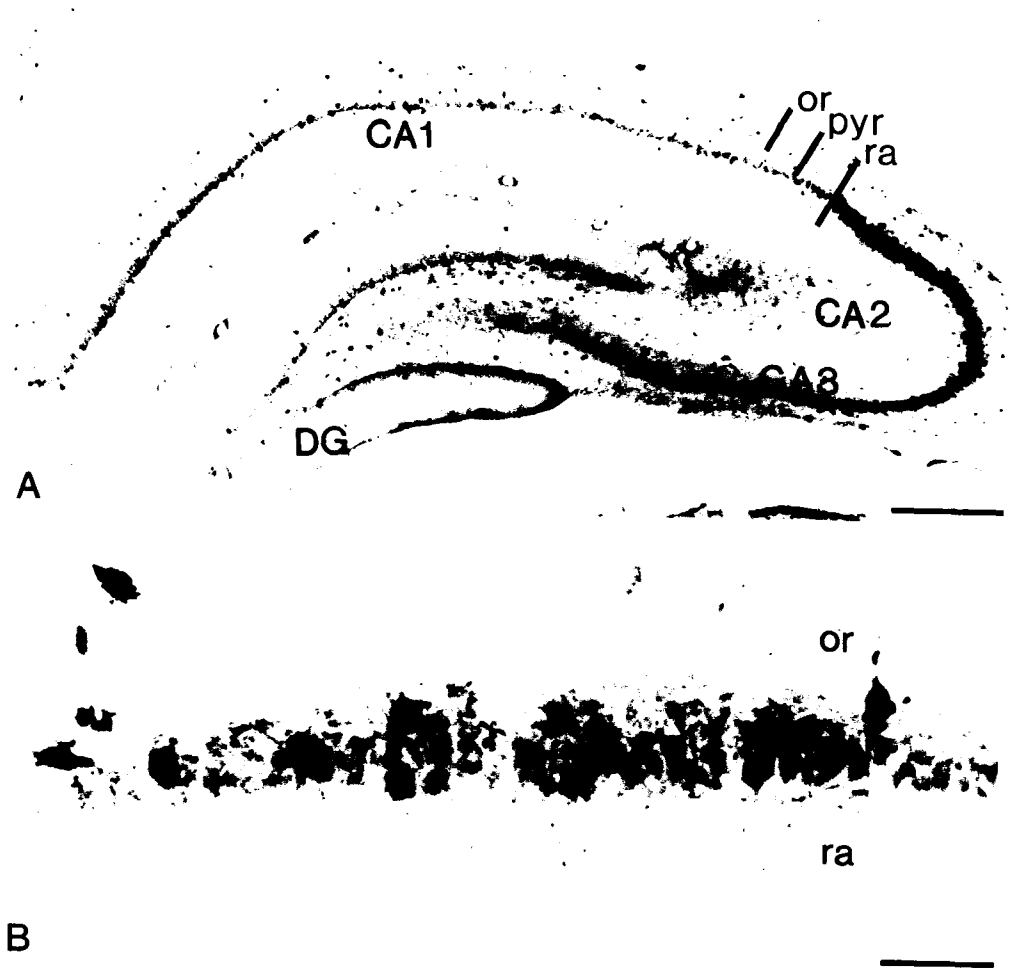

CA1
or
pyr
ra
CA2
CA3
DG
A
or
ra
B

Fig. 7. A: Distribution of PC5A-immunoreactivity in the mediodorsal thalamus. Intensely labeled neurons are evident throughout the laterodorsal (LD), paraventricular (PVN), and mediodorsal (MD) thalamic nuclei. Numerous PC5A-immunoreactive cells are also apparent within the intralaminar nuclei, including the centromedian nucleus (CM). B: Preadsorbed control. Section taken in a slightly more caudal plane and virtually label-free following incubation with PC5A antiserum preadsorbed with 90 μM antigenic peptide. Abbreviations: sm, stria medullaris thalami; 3V, third ventricle. Scale bar = 300 μm.

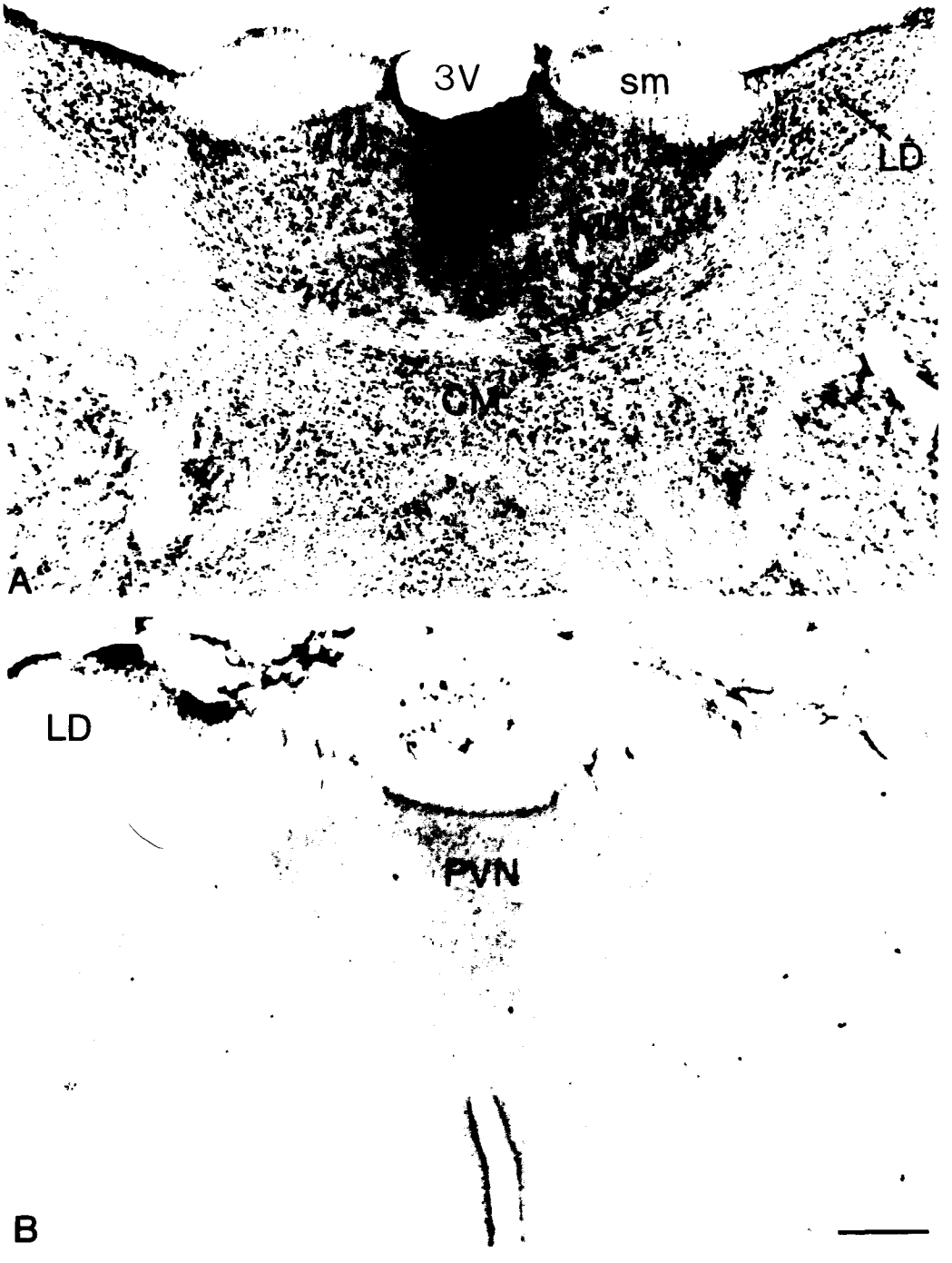
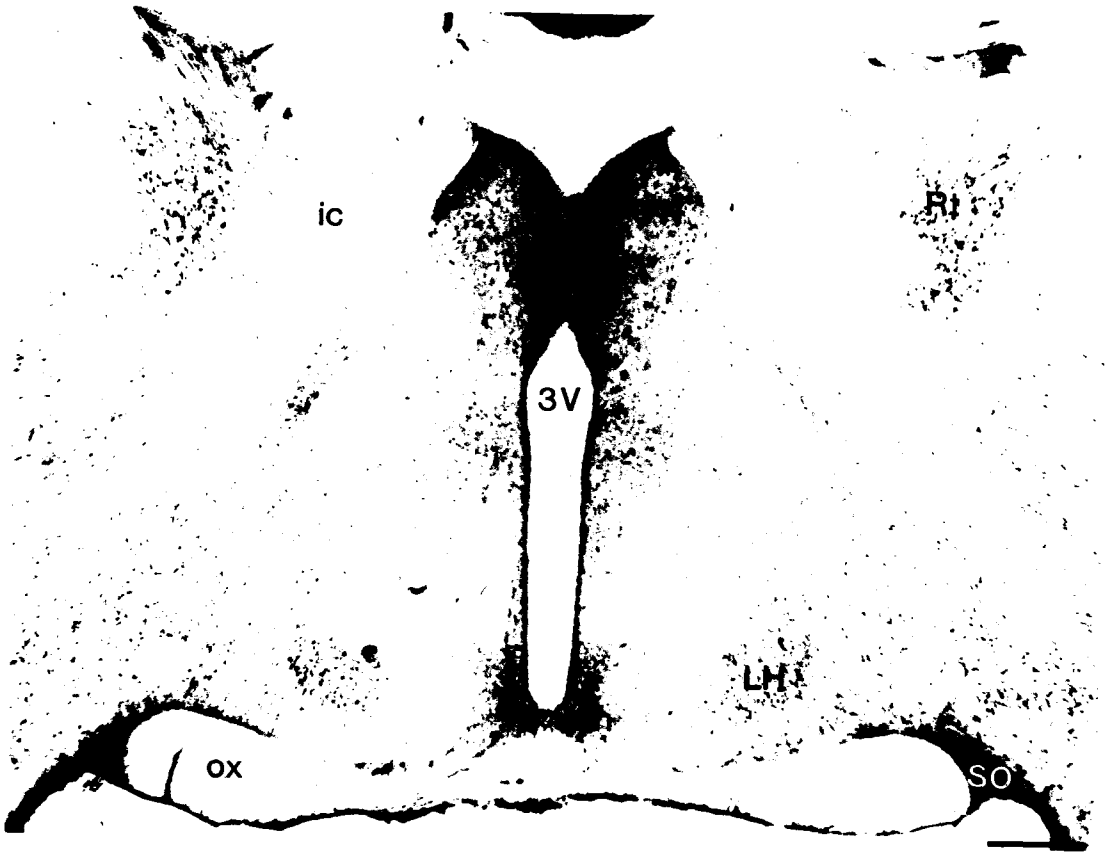

3V
sm
LD
CM
A
LD
PVN
B

Fig. 8. Distribution of PC5A immunoreactivity in the diencephalon. Intense immunoreactivity is evident in the supraoptic nucleus of the hypothalamus (SO) as well as in the paraventricular (PVN) and reticular (Rt) nuclei of the thalamus (PVN). The suprachiasmatic (Sch) and the anterior lateral hypothalamic nuclei (LH) are moderately labeled. Abbreviations: ox, optic chiasm; 3V, third ventricle; ic, internal capsule. Scale bar = 500 μm.


ic
Rt
3V
LH
ox
SO

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DISCUSSION

The present study provides the first description of the topographic and cellullar distribution of PC5A immunoreactivity in the adult rat brain. The antibody used in the present experiments was generated against the N-terminal DYDLSHAQSTYFNDPK sequence of the mouse enzyme which is identical, except for the Histidine 121 residue (substituted with an Arginine), to the rat 116-132 sequence (Basak et al., 1995). As such, the antibody recognizes both A and B splice variants of the rat enzyme, as well as the shorter, processed forms of each of these two isoforms (De Bie et al., 1996). In practice, however, the reaction product detected here is likely to correspond to PC5A derivatives, as Northern blotting experiments have shown PC5A to be the major form of PC5 mRNA expressed in rat brain (Lusson et al., 1993; Dong et al., 1995). Furthermore, Western blotting experiments carried out on rat brain extracts identified a 65kDA reaction product as the major form of immuno-detectable PC5 in the mediobasal hypothalamus (Dong et al., 1997). This protein, which was recognized in both AtT-20 and PC12 cells transfected with cDNA encoding the PC5A isoform as one of the two a mature forms of PC5A (De Bie et al., 1996; Barbero et al., 1998), is therefore likely to be the one visualized in the present study.

The applicability of the present antibody towards specific immunohistochemical detection of PC5A was previously demonstrated in AtT-20 (De Bie et al., 1996) and PC12 cells (Barbero et al., 1998). Both cell types showed intense immunostaining for the enzyme when transfected with cDNA encoding PC5A, but not when non transfected, as expected from their low content in endogenous PC5A (Seidah et al., 1994; Barbero et al., 1998). Further evidence for labeling specificity was provided here by extinction of the immunolabeling through preabsorption of the antibody with the immunogenic peptide.

The present results demonstrate that PC5A immunoreactivity is extensively, but selectively distributed in the rat forebrain. By and large, the distribution of PC5A-immunoreactive cells correlates well with that previously reported for PC5A mRNA in

the adult rat brain (Dong et al., 1995). Thus, high concentrations of both PC5A-immunoreactivity and PC5A mRNA were detected in the olfactory bulb, globus pallidus, lateral septum, diagonal band of Broca, iso and allocortex, hippocampal formation and hypothalamus. Furthermore, in all of these regions, there was a good correlation between the sub-regional distribution of immunolabeled and *in situ* hybridizing cells. For instance, in the olfactory bulb, both immunoreactive and hybridizing cells predominated in the mitral cell layer; in neocortex, both predominated in layer V; in the hypothalamus, both were enriched in the medial preoptic, supraoptic, paraventricular and arcuate nuclei; in the hippocampus, both were more intensely labeled in pyramidal cells of CA2/3 than of CA1 (Dong et al., 1995).

There were, however, a few areas in which the correlation between *in situ* hybridization and immunohistochemical signals was not as strong. Most notable among these were the neostriatum and the thalamus in which large numbers of moderately immunoreactive cells were detected but only sparse PC5A-hybridizing cells had been previously reported (Dong et al., 1995). These discrepancies cannot be attributed to non specific immunohistochemical labeling since preadsorption of the antibody with the cognate peptide was equally efficient at extinguishing the immunohistochemical signal in these as in other brain regions (e.g. Fig. 7). Furthermore, these areas were found to indeed express PC5A mRNA when analyzed by Northern blot (Dong et al., 1995). The observed differences therefore likely reflect true regional mismatches between mRNA and protein levels, as previously reported for a variety of proteins (Boudin et al., 1996; Morimoto et al., 1996; Schmitt et al., 1996).

A major finding of the present study was that in all regions examined, the bulk of neuronal PC5A immunoreactivity was associated with neuronal perikarya and proximal dendrites. Within nerve cell bodies, dual immunodetection of PC5A and of the medial cisternae Golgi marker MG-160 (Gonatas et al., 1995) demonstrated partial overlap of the

two antigens, indicating that a major fraction of the enzyme was concentrated within the Golgi apparatus. This finding is consistent with earlier observations in AtT-20 (De Bie et al., 1996) and PC12 cells (Barbero et al., 1998) in which PC5A was also found to be predominently associated with the Golgi complex. In addition, immunoreactive PC5A was detected here by both conventional and confocal microscopy within small punctate organelles peripheral to Golgi stacks, indicating that part of the enzyme is also located upstream (e.g., in the endoplasmic reticulum) or downstream (e.g., in secretory vesicles) of the Golgi. Taken together, these observations are congruent with the hypothesis that in neurons, as in transfected neuroendocrine cell lines (Barbero et al., 1998), PC5A is involved in the maturation of pro-peptides within the regulated pathway of secretion.

In this context, it was surprising to find that immunoreactive PC5A was not present in axon terminals, as demonstrated by the lack of co-localization of the PC5A antigen with the axon terminal marker, dynamin. Given that our antibody recognizes mature as well as unprocessed forms of the enzyme (De Bie et al., 1996; Dong et al., 1997), this observation suggests that PC5A is not stored and/or released with processed neuropeptides except in cases where these might be released from dendrites (Kombian et al., 1997). A similar interpretation was previously invoked to account for the lack of PC5A immunoreactivity in cell processes and for the minimal stimulation-induced release of the enzyme in PC12 cells transfected with cDNA encoding PC5A (Barbero et al., 1998). By contrast, in endocrine AtT-20 cells, PC5A was shown to accumulate extensively within mature secretory granules (De Bie et al., 1996). This suggests that the fate, and presumably also the function, of mature PC5A may vary between different cell types. It also implies that the processing of the PC5A precursor, and namely its truncation from the 117 kDa to the 65 kDa form, occurs earlier in the regulated secretory pathway of neurons than of endocrine cells (De Bie et al., 1996).

Dual immunostaining for PC5A and the astrocytic enzyme S-100α revealed that within the rat brain, PC5A immunoreactivity was never associated with astrocytes. This result complements those of earlier *in situ* hybridization studies, which had shown a predominant expression of PC5A in neurons, but did not afford the resolution necessary to exclude possible additional expression of the enzyme by glial cells. This exclusive association of PC5A with neurons is consistent with the purported involvement of this enzyme in neurosecretory processes in the brain (De Bie et al., 1996; Barbero et al., 1998).

The prominent localization of PC5A in regions of high neuropeptide contents, including the basal forebrain, lateral septum, bed nucleus of the stria terminalis, amygdala and hypothalamus documented in the present study is consistent with an involvement of this protein convertase in the processing of neuropeptide precursors. A prime candidate as a PC5A substrate is the precursor of oxytocin, since PC5A and oxytocin mRNA were found to be co-localized in neurons of the supraoptic and paraventricular nuclei of the hypothalamus (Dong et al., 1997). Another putative substrate is pro-neurotensin which is both processed by PC5A in transfected cells *in vitro* (Barbero et al., 1998) and expressed in several of the nuclei found here to contain PC5A including the lateral septum, bed nucleus of the stria terminalis and paraventricular nucleus of the hypothalamus (Woulfe et al., 1994). PC5A might also play a role in the processing of precursors of pro-corticotropin-releasing factor (CRF) and of pro-enkephalin, in view of the exceptionally strong and selective PC5A immunolabeling of presumptive CRF-containing mitral cells in the olfactory bulb and of large, putative enkephalinergic cells in the globus pallidus (Simantov et al., 1977; Imaki et al., 1989). The presence of immunoreactive PC5A in several brain nuclei previously documented to express BNDF in the adult rat brain (Conner et al., 1997; Friedman et al., 1998; Furukawa et al., 1998), together with the recent demonstration that BDNF can be sorted through the regulated secretory pathway

(Fawcett et al., 1997) and may be cleaved by PC5 *in vitro* (Seidah et al., 1996a), suggests that PC5 might also be involved in the biological processing of growth factors *in vivo*. Obviously, further *in vitro* processing and dual immunohistochemical studies are required to resolve these issues.

In conclusion, the present study demonstrates a selective association of the protein convertase PC5A with a sub-population of neurons within the rat forebrain. The distribution of these PC5A-immunoreactive cells overlaps extensively with that of other prohormone convertases (including PC1, PC2, PACE4 and furin; Schafer et al., 1993), in keeping with the notion that a combination of various protein convertases is responsible for the final maturation profile of any given neural substrate. Nonetheless, the distribution of PC5A-immunoreactive neurons is compatible with a selective involvement of this enzyme in the processing of specific neuropeptide precursors and possibly also of neurotrophins. Furthermore, the fact that the enzyme was found by confocal microscopy to be particularly enriched within proximal components of the neurosecretory pathway, suggests that it is involved in the early steps of the processing of its neural substrates.

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CONNECTING TEXT #1

In Chapter 2, we have demonstrated that PC5A exhibited a heterogeneous and widespread distribution throughout the rat brain. The selective association of the convertase with neurons in areas documented to express a variety of neuropeptides is consistent with a role of PC5A in the endoproteolytic conversion of numerous neuropeptide precursors. In particular, PC5A immunoreactivity was high in regions known to express large numbers of NT-immunoreactive nerve cell bodies (Jennes et al., 1982; Woulfe et al., 1994). These regions include the lateral septum, diagonal band of Broca, bed nucleus of the stria terminalis, medial preoptic area, arcuate and paraventricular nuclei of the hypothalamus, and paraventricular nucleus of the thalamus. In addition, PC5A can process the NT/NN precursor, at least *in vitro* (Barbero et al., 1998). PC2 is extensively distributed in the brain, where it is expressed exclusively by neurons and it processes very efficiently the NT/NN precursor *in vitro* (Rovere et al., 1996).

In this context, it was of interest to investigate the anatomical basis for a role of both PC2 and PC5A in the maturation of pro-NT/NN in the brain. We therefore propose in Chapter 3 a comparison of the immunohistochemical distribution of PC5A or PC2 with that of NT at the regional, cellular and subcellular levels.

Chapter Three

Immunohistochemical evidence for the involvement of protein convertase PC5A and PC2 in the processing of pro-neurotensin in rat brain

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IMMUNOHISTOCHEMICAL EVIDENCE FOR THE INVOLVEMENT OF PROTEIN CONVERTASES PC5A AND PC2 IN THE PROCESSING OF PRO-NEUROTENSIN IN RAT BRAIN

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ABSTRACT

The neuropeptides/neurotransmitters neurotensin (NT) and neuromedin (NN) are synthesized by endoproteolytic cleavage of a common inactive precursor, pro-NT/NN. *In vitro* studies have suggested that the prohormone convertases PC5A and PC2 might both be involved in this process. In the present study, we used dual immunohistochemical techniques to determine whether either one or both of these two convertases were co-localized with pro-NT/NN maturation products and could therefore be involved in the physiological processing of this propeptide in rat brain. PC2-immunoreactive neurons were present in all regions immunopositive for NT. All but three regions expressing NT were also immunopositive for PC5A. Dual localization of NT with either convertase revealed that NT was extensively co-localized with both PC5A and PC2, albeit with regional differences. These results strongly suggest that PC5A and PC2 may play a key role in the maturation of pro-NT/NN in mammalian brain. The regional variability in NT/PC co-localization patterns may account for the region-specific maturation profiles previously reported for pro-NT/NN. The high degree of overlap between PC5A and PC2 in most NT-rich areas further suggests that these two convertases may act jointly to process pro-NT/NN. At the subcellular level, PC5A was largely co-localized with the mid-cisternae Golgi marker MG-160. By contrast, PC2 was almost completely excluded from MG-160-immunoreactive compartments. These results suggest that PC5A, which is particularly efficient at cleaving the two C-terminal-most dibasics of pro-NT/NN, may be acting as early as in the Golgi apparatus to release NT, whereas PC2, which is considerably more active than PC5A in cleaving the third C-terminal doublet, may be predominantly involved further distally along the secretory pathway to release NN.

INTRODUCTION

Neuropeptides are synthesized as part of larger peptide precursors that are processed by endoproteolytic cleavage at specific dibasic sites by enzymes that belong to a family of subtilisin-like proteases. So far, eight of these proteases have been cloned and are referred to as PC1 (Seidah et al., 1990; Seidah et al., 1991) (also known as PC3; (Smeekens et al., 1991), PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990), furin (Roebroek et al., 1986; Barr et al., 1991), PACE4 (Kiefer et al., 1991), PC4 (Nakayama et al., 1992; Seidah et al., 1992a), PC5/6A and B (Lusson et al., 1993; Nakagawa et al., 1993), PC7 (Seidah, et al., 1996; also known as PC8 (Bruzzaniti et al., 1996)) or LPC (Meerabux et al., 1996), and SKI-1 (Seidah et al., 1999; see Seidah and Chretien, 1997, for review). With the exception of PC4, all these PCs were shown to be present in mammalian brain (Schafer et al., 1993; Dong et al., 1995; Seidah et al., 1996; Seidah et al., 1999).

In the case of PC1 and PC2, this localization is exclusive to neural and endocrine tissues (Schafer et al., 1993; Dong et al., 1995). Furthermore, PC1 and PC2, together with one of the two isoforms of PC5 (PC5A), are the only convertases to be sorted through the regulated secretory pathway (De Bie et al., 1996; Barbero et al., 1998), which makes them ideal candidates for the processing of neuropeptides. Indeed, *in vitro* studies in neuroendocrine cell lines expressing native or recombinant PC1, PC2 or PC5A have shown that these enzymes alone or in combination were capable of processing differentially pro-enkephalin (Mathis and Lindberg, 1992; Breslin et al., 1993; Johanning et al., 1998), pro-somatostatin (Brakch et al., 1995; Galanopoulou et al., 1995), pro-thyrotropin-releasing hormone (Schaner et al., 1997), pro-opiomelanocortin (Benjannet et

al., 1991; Seidah et al., 1992b; Friedman et al., 1994), pro-neurotensin (Rovere et al., 1996; Barbero et al., 1998), pro-dynorphin (Dupuy et al., 1994), pro-neuropeptide Y (Paquet et al., 1996; Brakch et al., 1997), pro-cholecystokinin (Wang et al., 1998), and pro-melanin concentrating hormone (Viale et al., 1999). However, the extent to which each of these PCs is actually involved in the physiological processing of these different neuropeptides *in vivo* is still unclear.

Neurotensin (NT) and neuromedin N (NN) are two structurally related peptides documented to play neurotransmitter/hormonal roles in both the brain and the periphery (see Kitabgi et al., 1992 for review). They are widely and heterogeneously distributed in rat central (Uhl et al., 1977; Jennes et al., 1982) and peripheral (Lundberg et al., 1982; Krukoff et al., 1985; Caverson et al., 1989) nervous systems and are highly concentrated in the gut (Carraway and Leeman, 1976). These two peptides are the maturation products of a common 169 amino-acid polypeptide molecule, pro-NT/NN (Dobner et al., 1987; Kislauskis et al., 1988). As shown in Figure 1, rat pro-NT/NN possesses four Lys-Arg dibasic residues. NN and NT are located at the C-terminal portion of the precursor and are separated from one another by the dibasic site Lys^{148}-Arg^{149}. The dibasic Lys^{163}-Arg^{164} is located at the C-terminal of NT, whereas the Lys^{140}-Arg^{141} doublet separates NN from the rest of the precursor. A fourth dibasic site is located at Lys^{85}-Arg^{86}. However, previous work from our laboratories has shown that this most N-terminal site is less extensively processed than the remaining three in the brain and is not cleaved at all in the gut (Carraway and Mitra, 1990; Kitabgi et al., 1991; de Nadai et al., 1994). Furthermore, in the gut, the third (Lys^{140}-Arg^{141}) dibasic site is only poorly cleaved so

that pro-NT/NN processing gives rise to large forms of NT and NN that are not found in the brain (Carraway and Mitra, 1990; Shaw et al., 1990; Carraway and Mitra, 1991).

Experiments performed on stably-transfected endocrine cells showed that PC1, PC2 and PC5A differentially processed pro-NT/NN (Rovere et al., 1996; Barbero et al., 1998). Thus, PC1 cleaved this pro-peptide with a pattern similar to that observed in the gut (Rovere et al., 1996). Accordingly, PC1 was shown by dual labelling immunohistochemistry to co-localize with pro-NT/NN in the endocrine N cells of the rat ileum suggesting that it is physiologically involved in pro-NT/NN processing in the gut (Barbero, 1999). PC2 generated equivalent amounts of NT and NN with very little of the large forms. This pattern of peptide production resembles that found in the brain, suggesting that PC2 might be a major pro-NT/NN convertase in this tissue (Rovere et al., 1996). However, detailed studies of pro-NT/NN processing in different brain structures revealed regional variations in the proportions of processing products (Kitabgi et al., 1991; de Nadai et al., 1993), implying that PC2 might not be the sole pro-NT/NN cleaving enzyme in the brain. Finally, PC5A cleaved pro-NT/NN with a pattern that differed from that of PC1 and PC2 and was reminiscent of that described in the adrenals (Barbero et al., 1998). However, PC5A was also found to be abundantly distributed in the brain, where its distribution resembled that of pro-NT/NN in a number of areas (Villeneuve et al., 1999).

In the present study we sought to investigate by dual labelling immunohistochemistry whether the cellular localization of PC2 and PC5A in rat brain was consistent with a role of either one or both of these enzymes in the physiological processing of the pro-NT/NN precursor in the CNS. We also examined the subcellular distribution of PC2, PC5A, NT

and the exposed Glu^{134}-Ile^{139} (E6I; Fig. 1) sequence of pro-NT/NN in order to determine whether the compartmentalization of enzymes and maturation products was in keeping with the maturation profile of the pro-NT/NN precursor documented *in vitro*.

MATERIALS AND METHODS

Antibodies

The PC5A antiserum was raised against the amino acid sequence 116-132 located within the N-terminal segment of mouse PC5A (Basak et al., 1995). The antibody used to detect PC2 was raised against amino acids 529-637 in the C-terminal segment of mPC2 (Seidah et al., 1990). Antibodies against NT raised in rabbit (Incstar; Stillwater, Mn) and rat (Eugene Tech; Ridgefield Park, NJ) were used for single and double labelling experiments, respectively. The site-directed E6I antibody was generated against the free C-terminal EKEEVI sequence extremity exposed after cleavage of the pro-NT molecule at the Lys^{139}-Arg^{140} dibasic residues (Bidard et al., 1993) (Fig. 1). Finally, the monoclonal antibody directed against rat MG-160, a constitutive protein of the medial cisternae of the Golgi apparatus (Gonatas et al., 1995), was a generous gift from Nicholas K. Gonatas (University of Pennsylvania).

Immunoblot analysis

Olfactory bulbs and hypothalami were pooled from 10 and 5 rat brains, respectively, and homogenized in 5 and 10 ml of 5 mM Tris buffer (TB), pH 7.4, respectively, using a polytron (3 X 10 seconds; setting 7-8). To one volume of extract was added one volume of phosphate buffer (PB; 50 mM Na_2HPO_4, pH 7.4) containing 1 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.1% triton X-100, 0.5% Nonidet P40, and 0.9% NaCl. The diluted extracts were centrifuged (20 minutes at 20 000 g) and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent, according to the procedure recommended by the manufacturer (Bio-Rad; Mississaauga, Canada).

Fifty μg of protein were subjected to Western blot analysis as previously described (Rovere et al., 1996). The PC5A and PC2 antisera were both used at a 1/1000 dilution. For control experiments, the PC5A antiserum was incubated for 2 h at 37 °C with 5μM synthetic antigen. PC2-expressing PC12 cells were extracted and immunoblotted as previously described (Rovere et al., 1996).

Colchicine administration

All animal procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee. For optimizing immunohistochemical detection of NT/E6I-containing nerve cell bodies, adult male Sprague-Dawley rats (260-280 g), purchased from Charles River Canada (St-Constant, Québec), were anaesthetized with sodium pentobarbital, 80 mg/kg i.p. (Somnotol; MCT Pharmaceuticals, Cambridge, Canada,), injected stereotaxically with 30 μl of colchicine (2 mg/ml) into the left lateral ventricle, and allowed to survive for 48 hours prior to sacrifice.

Tissue fixation

Untreated and colchicine-injected rats were deeply anesthetized with somnotol as above and fixed by transaortic perfusion of aldehydes. For PC5A immunolabelling, the animals (n = 10) were perfused with 500 ml of a mixture of 4 % paraformaldehyde and 0.2 % picric acid in 0.1 M PB, pH 7.4, at 4 °C. The brain was dissected out of the skull and post-fixed for 40 minutes by immersion in the same fixative. For PC2 immunolabelling, animals (n = 10) were perfused with the same fixative but at room

temperature and no post-fixation by immersion was carried out after the perfusion step. Both fixation protocols proved equally suitable for double-labelling experiments with either NT or MG-160 antibodies. For dual labelling experiments, rats were perfused according to the same protocol as for visualization of PC5A except in cases calling for the detection of PC2 in which the perfusion was performed according to the PC2 protocol. All fixed brains were cryoprotected for 24 hours by immersion in a 30 % sucrose solution in 0.2 M PB, pH 7.4 at 4 °C, frozen in isopentane at -50 °C, and sectioned at a thickness of 30 μm on a freezing microtome.

Single-labelling immunohistochemistry

Brain sections from colchicine-treated animals were collected in PB, pH 7.4. Two adjacent series, taken through the forebrain at 200μm intervals, were processed for single immunolabelling using antibodies against NT (Series 1) and either PC5A or PC2 (Series 2). Briefly, after two rinses in 0.1 M Tris buffer saline, pH 7.4 (TBS), sections were treated for 20 minutes with 0.3 % H_2O_2 in TBS to quench endogenous peroxidase. They were then blocked for 30 minutes at room temperature with TBS containing 3% normal goat serum (NGS) and incubated overnight at 4 °C in a TBS solution containing 1 % NGS, 0.2 % Triton X-100 and either PC5A (1:5000), PC2 (1:600), or NT (1:20 000) antibodies. As negative controls, sections were incubated with antisera pre-adsorbed with excess of the antigenic peptide or without primary antibodies. Following two rinses in TBS containing 1 % NGS, sections were incubated for 50 minutes at room temperature with biotinylated goat anti-rabbit immunoglobulins (1/100; Jackson ImmunoResearch, West Grove, PA) and then for 50 minutes with an avidin-biotin peroxidase complex

(ABC; Vector, Burlingame, CA). The sections were then rinsed twice in TBS, transferred to a TBS solution containing 0.1 % biotinylated tyramine (NEN-Dupont, Boston, MA) and 0.01 % H_2O_2 for 10 minutes, and reincubated for 50 minutes in the avidin-biotin peroxidase complex. Bound peroxidase was visualized by immersion of the sections in 0.01 M TB, pH 7.6, containing 0.05 % 3,3' diaminobenzidine, 0.04 % nickel chloride and 0.01 % H_2O_2. Finally, sections were mounted on gelatin-coated slides, dehydrated in graded ethanols, defatted in xylene, coverslipped and examined under a Leitz Aristoplan microscope.

Double-labelling immunohistochemistry

For cellular and sub-cellular identification of pro-NT processing compartments, the following pairs of antigens were co-visualized by fluorescence immunohistochemistry: (1) PC5A/NT; (2) PC2/NT; (3) PC5A/MG-160; (4) PC2/MG-160; (5) NT/MG-160; (6) E6I/MG-160; (7) NT/E6I. All experiments requiring visualization of NT and or E6I were performed in colchicine-pretreated animals. For experiments (1) - (4), sections were prepared as for single labelling experiments and blocked for 30 minutes by immersion in a 3 % NGS solution in TBS. They were then incubated overnight at 4 °C in a TBS solution containing 1 % NGS, 0.2 % Triton X-100 and a mixture of rat NT (diluted 1:1000) or mouse MG-160 (1:25) antibodies and of either PC2 (1:800) or PC5A (1:2000) antibodies. The following day, sections were rinsed in TBS containing 1 % NGS and incubated for 50 minutes in the same buffer containing a mixture of biotinylated goat anti-rabbit IgGs and FITC-tagged goat anti-rat (experiments 1 and 2) or goat anti-mouse (experiments 3 and 4) IgGs, both at a dilution of 1:100 (Jackson Immunoresearch).

Sections were then rinsed in TBS and incubated for 50 minutes in ABC solution, rinsed again in TBS, incubated for 10 minutes in a biotinyl-tyramine solution, and reincubated in ABC. After several buffer washes, sections were incubated for 50 minutes in a solution of Texas Red-conjugated streptavidin (1:300) (Jackson ImmunoResearch). For experiments (5) - (7), sections were preincubated in TBS containing 3 % NGS and incubated at 4 °C for 24 hours for (5) and (6) or 48 hours for (7) in TBS containing 1% NGS, 0.2% Triton X-100 and an antibody mixture of rabbit NT (1:1000) and mouse MG-160 (1:25) (5), of rabbit E6I (1:1500) and mouse MG-160 (1:25) (6) or of rat NT (1:2000) and rabbit E6I (1:1500) (7). Sections were then rinsed in TBS and incubated in a solution containing a mixture of Texas Red-tagged goat-anti-rabbit and FITC goat anti-rat for (5) and (7) or FITC-tagged goat anti-mouse for (6). For all double-immunofluorescence experiments, sections were then washed in buffer and mounted with Aquamount.

Double-labelled sections were analyzed by Confocal microscopy using a Zeiss Confocal laser scanning microscope (CLSM) equipped with a Zeiss inverted microscope and an argon/krypton laser (488 and 568 nm). Images were processed using the Carl Zeiss CLSM software (version 3.1). Images were acquired sequentially for the two fluorofores (FITC and Texas Red) by single optical plane acquisition averaged over 32 scans per frame. Color images were sharpened and adjusted for contrast and brightness using Adobe Photoshop 4.0 software (Adobe Systems, Inc., San Jose, CA).

In all experiments, the absence of cross-reactivity of the secondary antibodies was verified by omitting one or both of the primary antibodies during the overnight incubation. In the case of experiments involving the PC5A or PC2, specificity controls included preadsorbing the antibodies with their cognate immunogenic peptide.

RESULTS

Immunoloblot analysis

In membranes prepared from rat olfactory bulb and probed with our PC5A antibody, the immunoreactive signal was concentrated within a single band migrating at approximately 65 kDa (Fig. 2). This band was no longer evident after preadsorption of the antibody with an excess of antigen (Fig. 2). Based on our previous biochemical characterization (De Bie et al., 1996), this immunoreactive form represents the active form PC5A which has been autocatalytically truncated at the C-terminus immediately following the P-domain. In membranes from rat hypothalamus blotted with our PC2 antibody, two distinct bands were detected at the 66 and 75 kDa marks, respectively (Fig. 2). Sequence analysis previously demonstrated that the 75 and 66 kDa proteins are pro-PC2 and PC2 respectively (Benjannet et al., 1993). Neither of the bands was apparent in membranes probed with antibody preadsorbed with excess antigen (not shown) or in membranes from PC2-deficient PC12 cells (Fig. 2).

Immunohistochemical distribution of PC2, PC5A and NT

To investigate the possible involvement of PC2 and PC5A in the processing of pro-NT in rat brain, we compared the distribution of each convertase with that of immunoreactive NT in serial adjacent sections from the forebrain of colchicine-treated rats. Since PC5A and PC2 required different fixation procedures for optimal immunohistochemical detection, different brains had to be used for immunohistochemical processing of the two enzymes.

In order to ascertain that colchicine treatment did not affect the distribution of immunoreactive convertases, we first compared the distribution of PC5A and PC2 in colchicine-treated versus non colchicine-treated animals. In both normal and colchicine-treated rats, PC5A and PC2 antibodies gave rise to widespread, but selective labelling patterns. However, PC5A immunoreactivity was generally stronger and more discreetly localized throughout the CNS than that of PC2. Furthermore, whereas colchicine treatment affected neither the distribution nor the intensity of PC2-immunoreactive cell bodies, it did induce a selective increase in the labelling intensity of PC5A-immunoreactive cells in regions that contained NT-immunoreactive neurons (Figs. 3-8).

Throughout the CNS of naive animals, immunoreactive PC5A and PC2 were predominantly concentrated within neuronal cell bodies and proximal processes. However, PC2-immunoreactive signal was found considerably more distally than PC5A within dendrites and was occasionally seen in beaded fibres typical of varicose axons, which was never the case for PC5A. Furthermore, in rats treated with colchicine, PC5A immunostaining was more restricted to cell bodies than in untreated controls. This was not the case for PC2 which assumed the same intra-neuronal distribution with and without colchicine.

In series in which adjacent sections were immunostained for NT (all from colchicine-treated rats), the distribution of NT-immunoreactivity was considerably more selective than that of either of the two enzymes and closely corresponded to that described in earlier reports (Jennes et al., 1982; Woulfe et al., 1994). We restricted the present analysis to regions of the brain containing the bulk of NT-immunoreactive nerve cell bodies, which included the nucleus accumbens, lateral septum, caudate-putamen,

diagonal band of Broca, bed nucleus of the stria terminalis, preoptic area, paraventricular, periventricular, arcuate and lateral hypothalamic nuclei, paraventricular nucleus of the thalamus, central amygdaloid nuclei, substantia nigra, ventral tegmental area, periaqueductal grey and dorsal raphe nucleus (Table 1). All of these regions were immunopositive for both PCs (Table 1).

Rostrally, numerous NT cell bodies were scattered throughout the nucleus accumbens (not shown). By contrast, within the same region, PC5A-immunoreactive neurons were both less numerous and less intensely stained than NT-immunoreactive ones. PC2-immunopositive neurons were as numerous as NT-immunopositive cells and, like the latter, were scattered throughout the nucleus. NT-immunoreactive nerve cell bodies were also numerous in the rostral tier of the lateral septum where they co-distributed with both PC5A- and PC2-immunoreactive cell bodies throughout the dorsolateral extent of the nucleus (Fig. 3).

In the caudate-putamen, strongly NT-immunopositive neurons were scattered both medially, along the medial border of the lateral ventricle, and dorsally, beneath the corpus callosum (not shown). These labelled neurons were small, suggesting that they mainly corresponded to medium spiny neurons. Neurons labelled for PC5A were much more numerous than NT-immunoreactive ones and comprised both small and large neurons distributed throughout the nucleus. These neurons were particularly numerous and intensely labelled in regions containing NT-immunoreactive nerve cell bodies. They also were, however, consistently more numerous than NT neurons. Although less intensely labelled than PC5A-positive cells, PC2-immunoreactive neurons were as numerous as these, but more uniformly distributed throughout the nucleus.

In the diagonal band of Broca, sparse NT-positive cell bodies were distributed along the dorso-ventral axis of the nucleus. Within the same region, there was a higher number of PC5A- than of NT-immunoreactive neurons. PC2-immunoreactive nerve cell bodies were as sparsely distributed as NT-immunopositive cells (Table 1).

The lateral portion of the bed nucleus of the stria terminalis contained intensely stained and densely packed NT-immunoreactive neurons whereas the ventral portion of the nucleus contained fewer NT-immunopositive cells (not shown). Neurons immunopositive for PC5A were few in number and weakly labelled in both segments of the nucleus. A moderate number of PC2-positive neurons were scattered in the lateral portion of the bed nucleus of the stria terminalis. A smaller number was also evident in the ventral portion of the nucleus. Both PC2 and PC5A neurons were similar in shape and size to NT-immunoreactive neurons.

In the medial preoptic area, NT-immunoreactive neurons were numerous and intensely labelled, particularly within the ventromedial portion of the medial preoptic nucleus proper (Fig. 4). A few NT-immunoreactive cells were also visible in the lateral preoptic area (not shown). Within the medial preoptic area, the distribution of PC5A-immunoreactive cells mimicked that of NT-positive ones in that they also were more numerous and more intensely labelled in the ventral tier of the medial preoptic nucleus than in the adjacent medial preoptic area (Fig. 4). By contrast, PC2-immunolabelled cells were homogeneously distributed throughout the same region (Fig. 4). Both PC5A- and PC2-immunoreactive cells were also numerous in the lateral preoptic area.

In the central nucleus of the amygdala, NT-containing cell bodies were densely packed and heavily labelled (not shown). By contrast, PC5A- and PC2-immunolabelled cells were scarce and only weakly stained.

Within the thalamus, a conspicuous cluster of intensely immunoreactive NT neurons was evident within the paraventricular nucleus (Fig. 5). The distribution of PC5A-immunoreactive neurons was strikingly similar to that of NT-immunoreactive cells within the same structure (Fig. 5). PC2-immunoreactive neurons were also detected in the paraventricular nucleus, but in contrast to NT- and PC5A- positive cells, not more so than in surrounding nuclei (Fig. 5).

Within the hypothalamus, few strongly NT-positive neurons were detected in the periventricular nucleus. There were also a few PC5A- and PC2-immunoreactive cells within this structure. Intensely labelled NT neurons were also scattered throughout the lateral hypothalamus (Fig. 6). Within the same region, PC5A- and PC2-immunoreactive cells were more numerous than NT-positive ones, but showed a distributional pattern similar to that of NT-positive cells (Fig. 6). Numerous NT-immunoreactive neurons were detected in the parvocellular segment of the paraventricular nucleus. PC5A-immunoreactive cells were also detected within this nucleus, but were confined to the magnocellular region. PC2-immunoreactive cell bodies were distributed throughout the nucleus. Finally, the arcuate nucleus displayed several moderately labelled NT neurons. PC5A- and PC2-positive neurons were also identified within this structure, but in higher number than NT-positive neurons (Table 1).

Sparse, highly NT-immunoreactive neurons were scattered throughout the ventral tegmental area and pars compacta of the substantia nigra (Fig. 7). Within the same

regions, PC5A-positive neurons were similarly scattered in the ventral tegmental area, but were more numerous than NT-positive cells in the substantia nigra where they pervaded both the pars compacta and pars reticulata (Fig. 7). PC2-immunoreactive neurons were only weakly labelled and fewer in number than NT-positive cells in these areas (Fig. 7).

Numerous NT-positive neurons were scattered in the ventral and lateral portions of the periaqueductal grey matter (Fig. 8). Within the same region, PC5A-immunopositive neurons were more numerous than NT-positive ones but appeared to be especially enriched in NT-positive zones (Fig. 8). PC2-immunoreactive cells were more numerous and less selectively distributed than either NT- or PC5A-immunoreactive ones.

The dorsal raphe nucleus exhibited numerous, strongly labelled NT-positive neurons. Within the same region, PC5A neurons were more numerous but less intensely labelled than NT-immunoreactive ones (Fig. 8). Finally, PC2 neurons were as numerous as NT neurons, but only weakly labelled.

Dual immunolabelling of NT- and PC5A- or PC2- cells

In order to determine whether PC5A and/or PC2 co-localized with NT at the cellular level, dual fluorescent immunolabelling of NT and of either PC5A or PC2 was performed in regions expressing the highest concentrations of NT cell bodies and the results were analysed by confocal microscopy. Regions studied included the nucleus accumbens, lateral septum, diagonal band of Broca, lateral and ventral bed nucleus of the stria terminalis, medial preoptic area, central amygdala, paraventricular, periventricular, arcuate and lateral hypothalamic nuclei, substantia nigra, periaqueductal grey and dorsal raphe nucleus. In all regions examined, we found that a large number of NT-

immunoreactive cells co-localized either one of the two PCs. There was, however, a wide variation in the degree of co-expression between the different regions (Table 1). By and large, the proportion of NT cell bodies immunopositive for PC2 tended to be much more constant between brain regions than that of NT cells bodies immunoreactive for PC5A (Table 1).

Regions containing the highest proportion of NT-positive cells dually labelled for PC5A were the lateral septum (Figs. 9 A-A"), ventral and lateral portion of the bed nucleus of the stria terminalis, lateral and medial preoptic areas, lateral hypothalamus, substantia nigra, and dorsal raphe. Virtually no neurons dually stained for PC5A and NT were detected in the nucleus accumbens or in the paraventricular (Figs. 9 B-B") and periventricular nuclei of the hypothalamus. Despite the relatively large number of PC5A- and NT-immunopositive neurons in the central amygdala, only a small proportion of the later co-localized PC5A. A moderate number of NT-positive neurons in the periaqueductal grey were immunofluorescent for PC5A.

All regions listed above also contained a sub-population of NT-immunoreactive cells which stained positively for PC2. The central amygdala, lateral hypothalamus, periaqueductal grey and dorsal raphe nucleus exhibited the highest proportions of NT-cell bodies that were co-stained for PC2. The nucleus accumbens, lateral septum, caudate/putamen, lateral and ventral portions of bed nucleus of the stria terminalis (Figs. 9 C-C"), medial preoptic area, paraventricular nucleus of the thalamus, paraventricular and periventricular hypothalamic nuclei (Fig. 9 D-D"), ventral tegmental area and substantia nigra pars compacta all contained a moderate number of PC2-positive neurotensinergic neurons.

Dual immunolocalization of PC5A, PC2, NT and E6I with the integral Golgi protein MG-160

In order to determine whether NT and/or its precursor were localized within the same subcellular compartments as PC5A and/or PC2, the intra-perikaryal distribution of NT, E6I, PC5A and PC2 were examined by confocal microscopy and compared to that of the mid-Golgi cisternae protein MG-160. Single confocal optical sections through PC5A-immunolabelled cells revealed that PC5A immunoreactivity predominated within, but also extended beyond, a juxtanuclear compartment co-localizing MG-160 (Figs. 10 A-A"). By contrast, PC2 immunoreactivity was diffusely distributed throughout neuronal perikarya and was hardly detectable within the MG-160-immunopositive compartment (Figs. 10 B-B"). NT immunoreactivity was mainly concentrated within small "hot spots" distributed throughout the cytoplasm of the cells as well as within larger juxta-nuclear patches (Fig. 10 C). NT immunoreactivity partly co-localized with, but largely exceeded that of MG-160 (Fig. 10 C'). Like NT, E6I immunoreactivity was concentrated within small intra-cytoplasmic puncta scattered over the cytoplasm of both perikarya and dendrites (Fig. 10 D) and only marginally co-localized with MG-160 (Fig. 10 D').

Co-localization of pro-NT/NN processing products

As previously reported (Woulfe et al., 1994), the distribution of E6I immunolabelling was essentially the same as that of NT immunoreactivity detected in either single or dual labelling experiments. Furthermore, by confocal microscopy, it was apparent that virtually all E6I-immunoreactive cells also contained NT immunoreactivity. Analysis of

the intracellular distribution of NT and E6I revealed extensive co-localization of the two antigens in neuronal perikarya, dendrites, and axon terminals. 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 or axon terminals. However, it was high enough to allow detection of an intracellular compartmentalization of the immunofluorescent labelling at the level of the perikarya and proximal dendrites. This compartmentalization took the form of small intensely fluorescent NT and E6I immunoreactive puncta heterogeneously distributed throughout the cytoplasm of the cells (Figs. 10 E-E'). Merging of individually processed images for NT and E6I immunolabelling demonstrated that all of these puncta were dually stained for both antigens, as indicated by the emission of yellow fluorescence (Fig. 10 E').

DISCUSSION

The present study provides anatomical evidence for the involvement of prohormone convertases PC5A and PC2 in the physiological endoproteolytic maturation of the pro-NT/NN precursor in mammalian brain.

The specificity of the antibodies used here for immunohistochemical detection of PC5A and PC2 has been extensively discussed elsewhere (Benjannet et al., 1993; Marcinkiewicz et al., 1993; Dong et al., 1997; Villeneuve et al., 1999). By Western blot, our PC5A antibody recognized a single specific band migrating at 65 kDa in extracts from the olfactory bulb and the hypothalamus, two regions known to express high levels of PC5A immunoreactivity (Villeneuve et al., 1999). A protein of the same size was also detected in samples from the paraventricular and supraoptic nuclei of the hypothalamus with this same antibody (Dong et al., 1997). Studies on transfected cell lines have shown that this 65 kDa form of PC5A corresponded to a C-terminal truncated form of the protease which, by analogy with a similarly mature form of PC1, was assumed to be the most enzymatically active form of PC5A (De Bie et al., 1996). Curiously, this 65 kDa form is only weakly expressed in AtT-20 and PC12 cells transfected with the cDNA encoding PC5A (the bulk of the enzyme being detected as 126 and 117 kDa forms; De Bie et al., 1996; Barbero et al., 1998), but found in abundance in αTC1-6 and STC-1 cells in which PC5A is expressed endogenously (Blache et al., 1994). These observations, together with the present data, suggest that PC5A is processed differently in systems expressing the enzyme endogenously versus ectopically and that 65 kDa is the size of the active enzyme in the brain.

In the case of PC2, our antibody specifically recognized by Western blot two distinct bands migrating at the 75 kDa and 68 kDa molecular weight marks in extracts from rat hypothalamus. Similar forms of the convertase were previously detected in various mammalian tissues including brain (Dong et al., 1995) as well as in cells transfected with cDNA encoding PC2 (Rovere et al., 1996). As in the present study, the 66 kDa protein was found to be more abundant than the 75 kDa one, in keeping with the notion that these proteins correspond to mature and precursor forms of the enzyme, respectively (Benjannet et al., 1993; Rovere et al., 1996).

The topographic distribution of NT-immunoreactive nerve cell bodies and axon terminals reported in the present study closely conformed to that previously reported in the brain of colchicine-treated rats (Jennes et al., 1982; Woulfe et al., 1994). Similarly, the distribution of PC2 and PC5A immunoreactivity broadly conformed to that previously reported for these two enzymes in the brain of noncolchicine-treated animals (Villeneuve et al., 1999; Winsky-Sommerer et al., 2000). The distribution of PC5A and PC2 immunoreactive cell bodies was also comparable (with some exceptions discussed in Villeneuve et al., 1999) with that of neurons shown to express PC5A and PC2 mRNA, respectively (Schafer et al., 1993; Dong et al., 1995). Accordingly, both PC5A and PC2 immunoreactive signals were completely abolished when the antibodies were preadsorbed with their respective cognate protein.

Comparison between the distribution of NT and of either PC5A or PC2 immunoreactivity in serial adjacent single-labelled sections showed extensive overlap between NT/PC5A and NT/PC2 labelling. However, the distribution of both PC5A and

PC2 immunoreactivity extended well beyond regions containing NT, in keeping with the more extensive distribution reported for the former than for the latter.

At the regional level, all nuclei containing NT-immunopositive nerve cell bodies expressed at least one of the two PCs and in most instances both of them. In the case of PC2, the density of immunoreactive cells found within regions containing NT-immunopositive neurons was not qualitatively different from that found within surrounding NT-immunonegative regions. By contrast, in the case of PC5A, the number of immunoreactive neurons was usually higher in regions containing NT-immunoreactive cells than in surrounding nuclei, suggesting a preferential concentration of the enzyme within these areas. Furthermore, the intensity of PC5A immunostaining was higher within than outside these regions. However, these higher levels of PC5A immunoreactivity were mostly observed in colchicine-treated rats, suggesting that there might be a linkage between the expression of the enzyme and that of the peptide.

At the cellular level, dual immunolocalization studies revealed that in all regions containing NT-immunoreactive nerve cell bodies, a proportion of the latter also stained positively for PC2. Similarly, in all but three of these regions (the nucleus accumbens and the para- and periventricular nuclei of the hypothalamus), a number of NT neurons stained positively for PC5A. Interestingly, two of the regions in which PC5A and NT did not co-localize, the paraventricular and the periventricular nuclei of the hypothalamus, do not express NT in animals non-treated with colchicine (Ceccatelli et al., 1991; Kiyama and Emson, 1991). This observation suggests that PC5A is only present in NT neurons that physiologically express the peptide. Within each NT-expressing region, the number of NT neurons dually labelled for either PC5A or PC2 was highly variable. This

variability was not related to the number of PC5A and PC2 neurons, as some PC5A-rich areas, such as the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus, or PC2-rich areas, such as the lateral septum and paraventricular nucleus of the hypothalamus showed only a small number of dually labelled neurons. It therefore appears that the variability in the extent of NT/PC co-localization reflects true physiological differences in the co-expression of the enzymes and their putative substrates.

The extensive co-localization of NT and PC2 and/or PC5A immunoreactivity reported in the present study provides strong anatomical evidence for a role of either or both of these convertases in the processing of pro-NT/NN. Indeed, when co-expressed with the precursor of NT in neuroendocrine cells, both PC5A and PC2 were found to efficiently cleave the two distal C-terminal dibasic residues, thereby releasing NT from its large precursor fragment (Rovere et al., 1996; Barbero et al., 1998). PC2 was also shown to efficiently process the Lys^{140}-Arg^{141} dibasic site of pro-NT/NN (Rovere et al., 1996), thereby giving rise to the same small form of NN as produced in the brain (de Nadai et al., 1994; Woulfe et al., 1994).

The fact that in certain brain regions such as the lateral hypothalamus, ventral tegmental area, periaqueductal grey and dorsal raphe, equally large numbers of NT-immunoreactive cells co-localized each of the two convertases further suggests that in many NT cells, PC5A and PC2 may act in concert to process pro-NT/NN. A similar type of cooperativity between PC1 and PC2 was previously proposed to account for the physiological maturation profile of insulin (Bennett et al., 1992; Smeekens et al., 1992) and pro-opiomelanocortin (Benjannet et al., 1991; Zhou et al., 1993) in endocrine tissues.

Such joint action is unlikely to be occurring in all NT neurons, however, since some regions, such as the nucleus of the central amygdala and the paraventricular nucleus of the hypothalamus, exhibited numerous NT/PC2 dually labelled cells but only few NT/PC5A double-labelled ones and others, such as the lateral septum and the diagonal band of Broca, showed high NT/PC5A but moderate or little NT/PC2 co-localization. These findings are congruent with reports of *in vitro* studies, showing that pro-NT/NN can be processed by PC2 in a fashion comparable to that seen in the brain in the absence of PC5A (Rovere et al., 1996), and in PC2 gene knock-out mice, which have shown that pro-NT/NN can be processed in the absence of PC2, albeit to a lesser extent than in normal mice (Barbero, 1999).

In certain regions, such as the nucleus accumbens and periventricular nucleus of the thalamus, there were only a few cells in which NT co-localized with either PC5A or PC2, suggesting that the NT/NN precursor can also be processed in the absence of either PC5A or PC2. A possible candidate for this case figure is PC1 which is selectively expressed in neuronal and endocrine cells and is extensively distributed in the brain (Schafer et al., 1993; Winsky-Sommerer et al., 2000). In any case, the participation of other prohormone convertases in the maturation of pro-NT/NN must be invoked to account for the lack of maturation of the N-terminal-most Lys^{85}-Arg^{86} doublet, which is partially cleaved in the brain, but is sensitive to neither PC1, PC2 nor PC5A *in vitro* (Rovere et al., 1996; Barbero et al., 1998). There is therefore likely to be a variable involvement of different PCs or cocktail of PCs in the maturation of the NT/NN precursor in the brain accounting for the reported regional variations in the relative proportions of processing products (Kitabgi et al., 1991; de Nadai et al., 1994; Woulfe et al., 1994).

Dual localization of PC5A or PC2 with the mid-cisternae Golgi marker MG-160 demonstrated that whereas PC5A was predominantly localized within the Golgi apparatus and to a lesser extent within more distal intraperikaryal compartments, PC2 was only marginally concentrated in the Golgi and predominated within extra-Golgian zones of perikarya and dendrites. These observations are congruent with localization data in various neuroendocrine cell lines (Lindberg et al., 1994; Malide et al., 1995; Barbero et al., 1998) and non-colchicinized rat brain (Villeneuve et al., 1999) and suggest that PC5A may be already active within "proximal" regions of the neuro-secretory pathway whereas PC2 may be most active more distally, within mature secretory granules. Initiation of pro-peptide processing in early compartments of the regulated secretory pathway was previously demonstrated in neurons for pro-somatostatin (Lepage-Lezin et al., 1991), aplysia's pro-egg-laying hormone (Sossin et al., 1990), and pro-thyrotropin-releasing hormone (Nillni et al., 1993). In the case of pro-NT/NN, such a mode of action could account for the sequential processing of the precursor previously postulated on the basis of the pattern of distribution of pro-NT/NN maturation products in central neurons (Woulfe et al., 1994). According to this scheme, the C-terminal most dibasics (Lys^{163}-Arg^{164} and Lys^{148}-Arg^{149}), which are preferentially cleaved by PC5A (Barbero et al., 1998), would be processed first to release NT and the third dibasic (Lys^{140}-Arg^{141}), which is cleaved with high efficiency by PC2 but not by PC5A, would be processed subsequently to release NN, thereby exposing the E6I epitope sequence. Not only would this type of sequential maturation sequence be consistent with the sub-cellular distribution of PC5A and PC2, but it would also account for the extensive co-localization

of NT and E6I within distal vesicular organelles, which suggests that the packaging in secretory granules precedes the cleavage of the Lys^{140}-Arg^{141} dibasic residues.

In conclusion, the present data suggest that both PC5A and PC2 are implicated in the physiological maturation of the NT/NN precursor. They are also consistent with a concerted action of both enzymes whereby PC5A would be responsible for the early processing of pro-NT/NN in proximal compartments of the regulated secretory pathway and PC2 for subsequent more distal processing leading to the sequential maturation of NT and NN.

TABLE, FIGURES AND FIGURE LEGENDS

Table 1: Co-localization of NT and PC5A or PC2 in rat brain

Region	NT*	PC5A*	NT/PC5A†	PC2*	NT/PC2†
Nucleus accumbens	+++	+/-	-	+++	++
Lateral septum	+++	+++	+++	+++	++
Caudate/putamen	+	+++	+	+++	++
Diagonal band of Broca	+	++	++	+	+
Bed nucleus of the stria terminalis, lateral	++++	+	+++	+	++
Bed nucleus of the stria terminalis, ventral	+++	+	++	+	++
Medial preoptic area	+++	+++	+++	+/-	++
Lateral preoptic area	+/-	+/-	+++	+	+
Central amygdala	++++	++	+	+	+++
Paraventricular nucleus, thalamus	+++	+++	+	++	++
Periventricular, hypothalamus	+/-	+/-	-	+/-	++
Lateral hypothalamus	++	+++	+++	+++	+++
Paraventricular nucleus, hypothalamus	+++	+++	-	++	++
Arcuate nucleus, hypothalamus	++	+++	+	+++	++
Ventral tegmental area	+	+	++	+/-	++
Substantia nigra, pars compacta	+	++	+++	+/-	++
Periaqueductal grey	+++	+++	++	++++	+++
Dorsal raphe	++	+++	+++	++	+++

* Semiquantitative estimates of the number of NT, PC2, and PC5 immunoreactive cells: -, not detectable; +/-, very low; +, low;++, moderate; +++, high, ++++, very high.

†Semiquantitative estimates of the number of neurons co-localizing NT and either PC2 or PC5:-, not detectable; +, low; ++, moderate; +++, high; ++++.

Figure 1: Schematic representation of rat pro-NT/NN and of its various maturation products. The position of the four Lys-Arg dibasic sites are illustrated along with NT, NN, and the peptide segment E6I exposed after proteolytic cleavage at the Lys^{140}-Arg^{141} dibasic site.

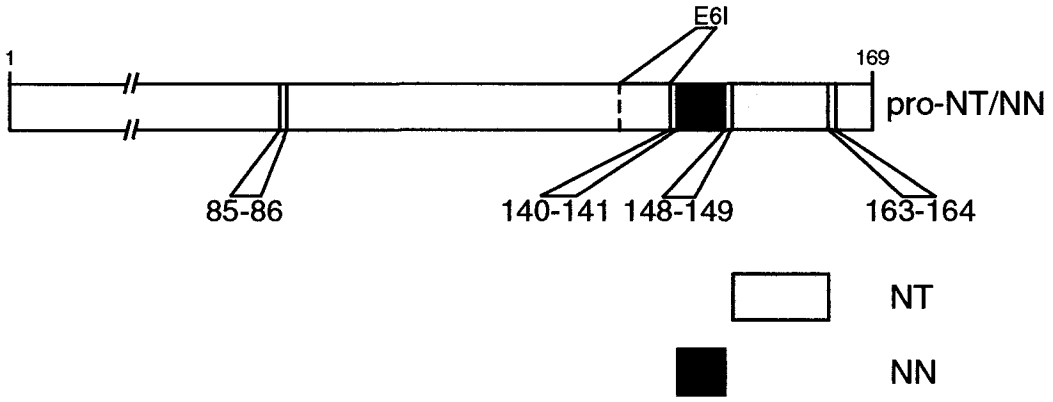

E6I
1
169
pro-NT/NN
85-86
140-141
148-149
163-164
NT
NN

Figure 2: Western blot analysis of PC5A and PC2 immunoreactivity in the olfactory bulb and hypothalamus, respectively. Proteins (50μg) from either rat olfactory bulb or hypothalamus extracts were separated on SDS-PAGE. After transfer to nitrocellulose, the proteins were immunoblotted with the PC5 and PC2 antisera used at a 1:1000 dilution. For PC5A control, the antibody was preadsorbed with 5 μM of cognate peptide. For PC2 control, extracts from PC12 cells, which are known to be devoid of endogenous PC2, were processed in parallel. The PC5A antibody recognized a single band migrating at 65 kDa. The PC2 antibody recognized 2 proteins migrating at 75 kDa and 66 kDa, respectively.

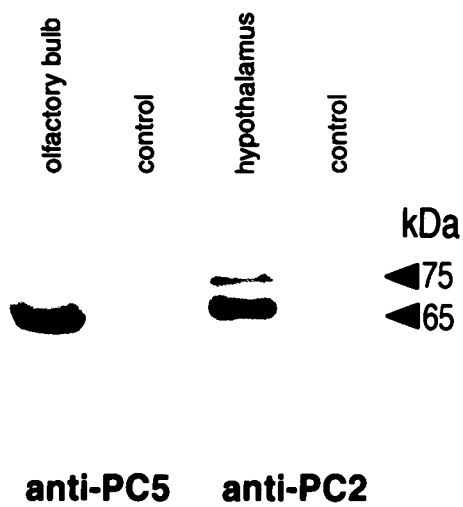

olfactory bulb
control
hypothalamus
control
kDa
75
65
anti-PC5
anti-PC2

Figure 3: NT, PC5A, and NT immunoreactivity in the lateral septum (LS). Note the regional overlap between NT, PC5A and PC2 immunoreactivity throughout the dorsal-lateral extent of the nucleus. 2V, lateral ventricle; cc, corpus callosum. Scale bar = 135 μm.

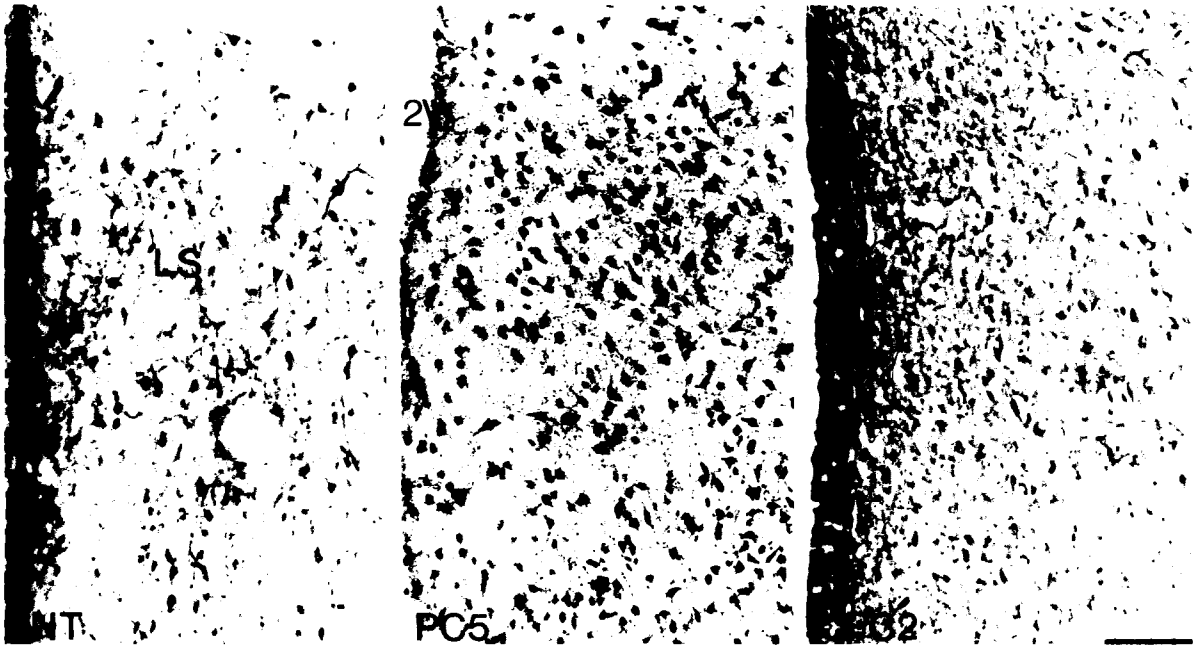

2V
LS
NT
PC5
PC2

Figure 4: Distribution of NT, PC5A, and PC2 immunoreactivity in the medial preoptic area (MPA). NT-immunoreactive cells form a tight cluster within the ventral segment of the medial preoptic nucleus (MPO). Only a few NT-immunopositive cell bodies are detected more laterally and more dorsally. PC5A immunoreactivity is similarly preferentially concentrated within the ventral portion of the MPO, partially overlapping with NT immunolabelling. By contrast, PC2-immunopositive neurons are distributed throughout the MPA without any obvious concentration at the level of the MPO. 3V, third ventricle; Pe, periventricular nucleus. Scale bar = 160 μm.

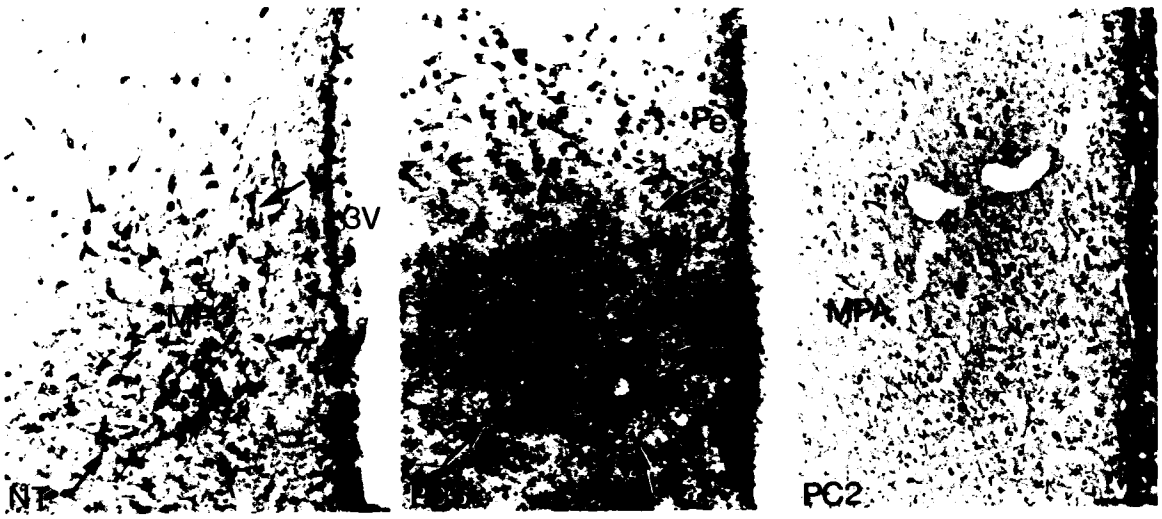

3V
Pe
MPA
PC2

Figure 5: NT, PC5A, and PC2 immunoreactivity in the paraventricular nucleus of the thalamus (PVN). Whereas NT and PC5A antibodies give rise to intense immunostaining concentrated within the PVN, PC2 immunoreactive cell bodies are less densely labelled and are not more numerous in the PVN than in surrounding nuclei. T3, choroid plexus of third ventricle; MD, mediodorsal nucleus of the thalamus. Scale bar = 163 μm.

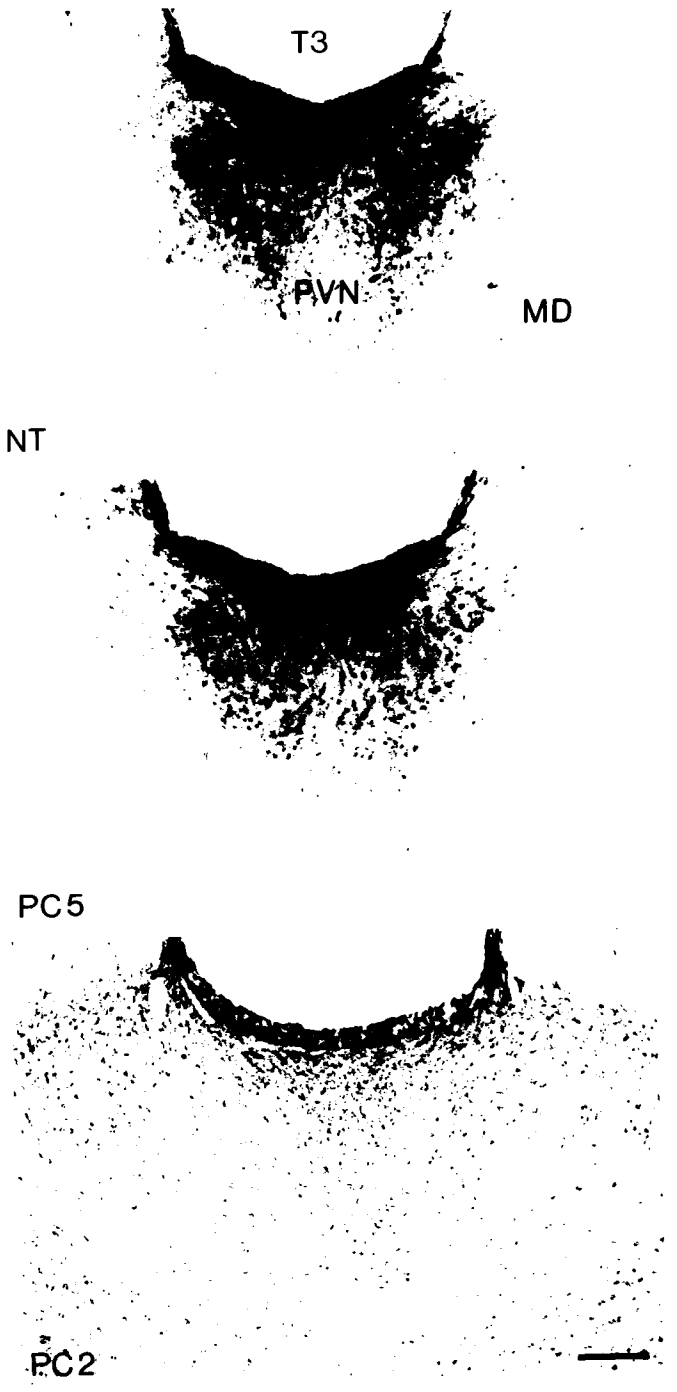
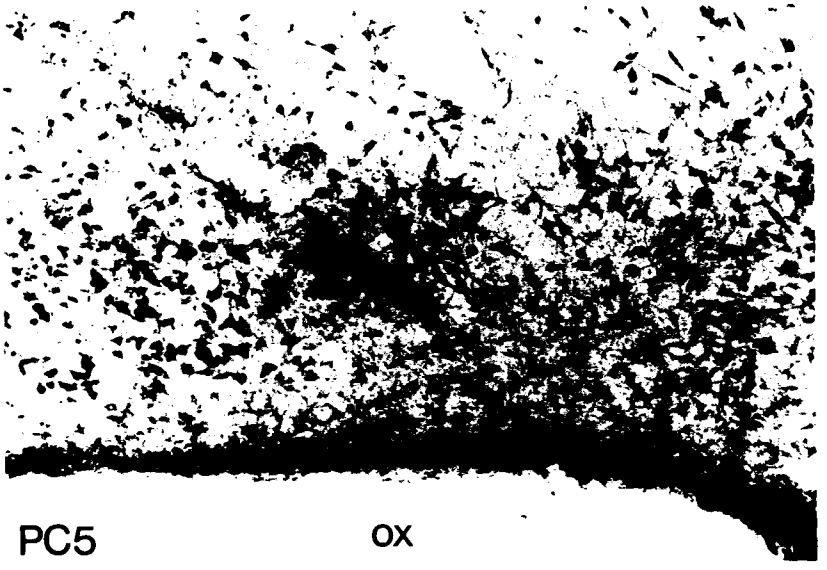

T3
PVN
MD
NT
PC5
PC2

Figure 6: NT, PC5A, and PC2 immunolabelling in the lateral hypothalamus (LH). NT-immunoreactive neurons are scattered within the ventral portion of the lateral hypothalamus, dorsal to the optic chiasm (ox). Both PC5 and PC2 immunopositive neurons are also present within the same region but to a considerably greater extent. Scale bar = 125 μm.

NT ox



PC5 ox

PC2 ox

Figure 7: Distribution of NT, PC5A and PC2 immunoreactivity in the substantia nigra and ventral tegmental area (VTA). NT immunoreactive cells are evident throughout the VTA and substantia nigra, pars compacta (SNC). PC5A immunoreactive cell bodies are mainly concentrated in the SNC but are also found throughout the VTA and substantia nigra, pars reticulata (SNR). PC2-immunoreactive cells are scarce, and mainly found within the SNR. Scale bar = 265 μm.

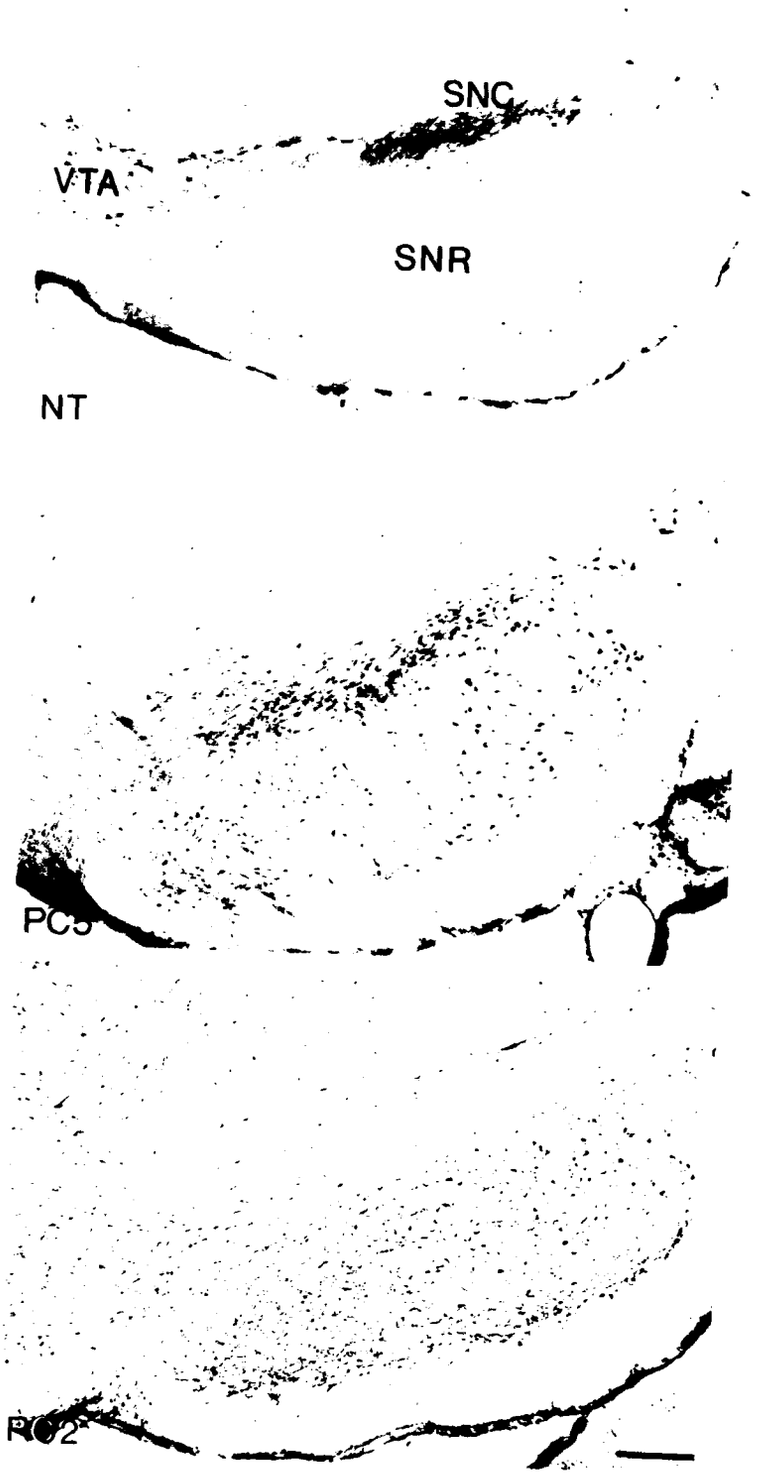

SNC
VTA
SNR
NT
PC5
RO2

Figure 8: Distribution of NT and PC5A immunoreactivity within the periaqueductal grey (PAG). Superposable patterns of intense NT and PC5A immunorectivity are apparent in the ventro-lateral portion of the PAG. A small number of neurons immunopositive for either antigen is also detected in the dorsal raphe nucleus (DR). Aq, aqueduct of Sylvius. Scale bar = 440 μm.

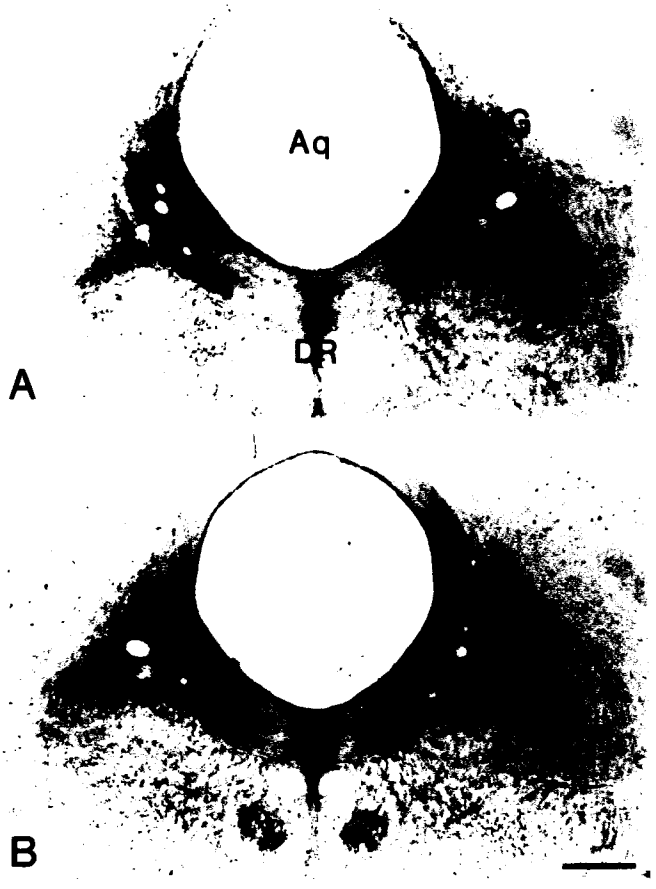

Aq
DR
A
B

Figure 9: Dual PC5A (A,B) or PC2 (C, D) and NT (A', B', C', D') immunostaining. Single confocal optical sections. In the lateral hypothalamus (A, A', A''), most NT-immunoreactive neurons (A') are also immunopositive for PC5A (A). These dually labelled cells stand out in yellow when images (A) and (A') are fused (A''). Only a few NT positive cells are PC5A negative (arrows), but several PC5A immunoreactive neurons are devoid of NT. In the paraventricular nucleus of the hypothalamus, PC5A-immunoreactive neurons are confined to the magnocellular segment (B, PVm) and NT-immunoreactive cells to the parvocellular segment (B', PVp). Note that the 2 populations are mutually exclusive (B''). In the lateral portion of the bed nucleus of the stria terminalis (C, C', C'') and paraventricular nucleus of the hypothalamus (D, D', D''), most NT immunopositive neurons co-localize PC2 (C'', D''). Scale bar = 75 μm.

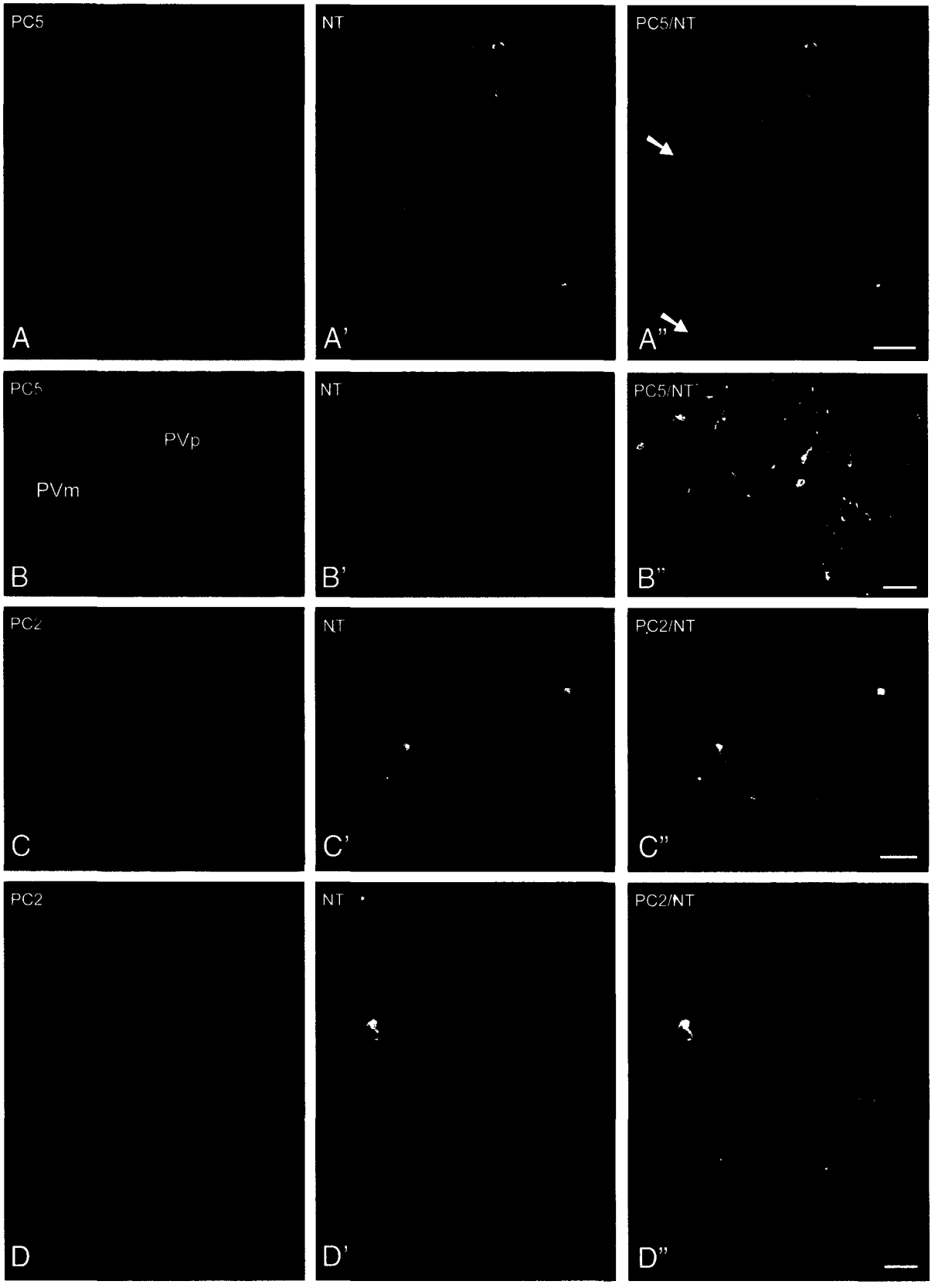
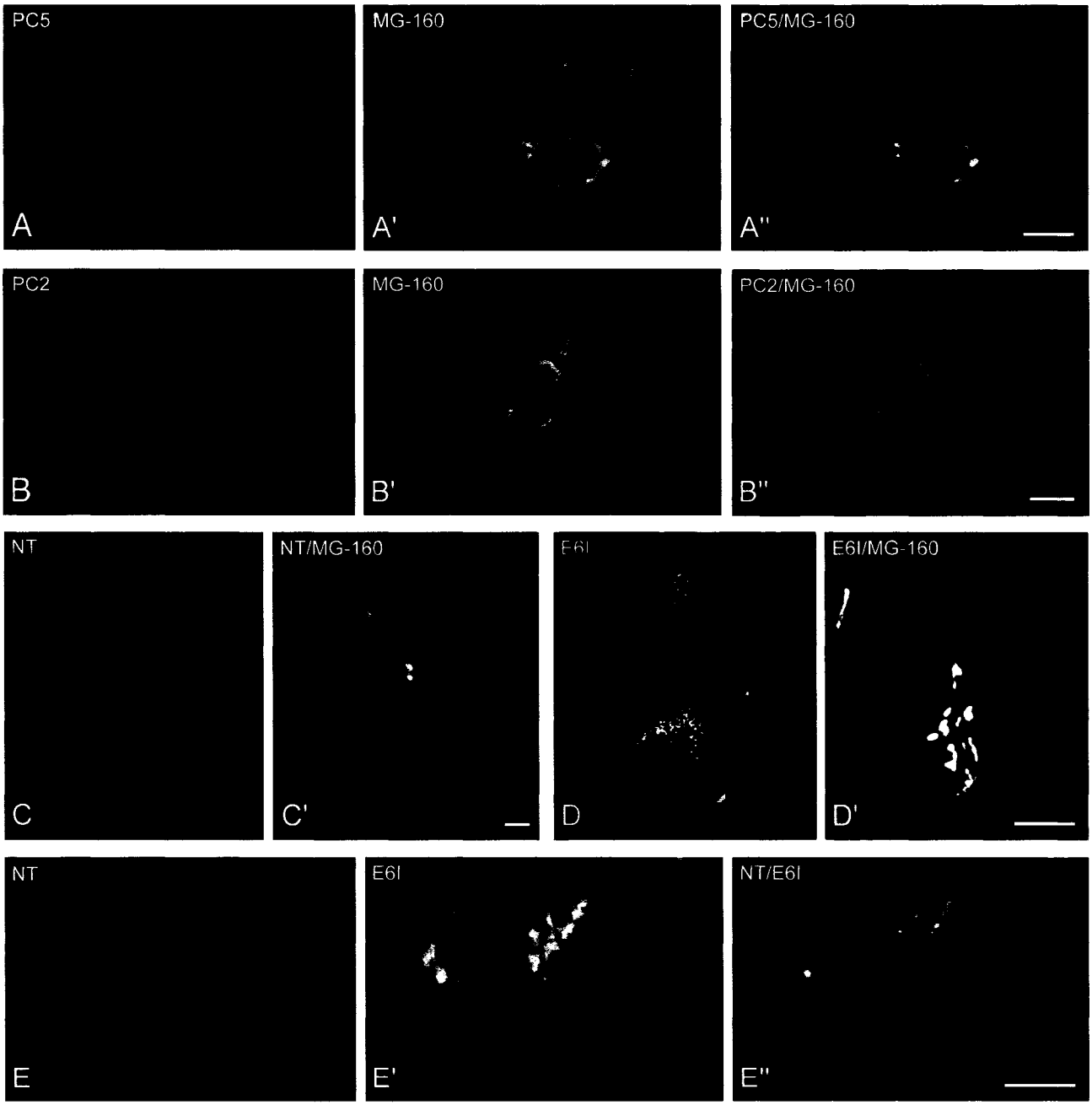

PC5
NT
PC5/NT
A
A'
A"
PC5
PVp
PVm
NT
PC5/NT
B
B'
B"
PC2
NT
PC2/NT
C
C'
C"
PC2
NT
PC2/NT
D
D'
D"

Figure 10: A, B: Confocal microscopic images of cortical neurons labelled for PC5A (A) or PC2 (B) and the Golgi protein MG-160 (A', B'). Merging of (A) and (A') demonstrates that the bulk of PC5A immunoreactivity is concentrated in the same intracellular compartment as MG-160 (A''). The pattern is different for PC2 which appears to be excluded from the MG-160-immunopositive compartment (B''). C, D: NT-immunopositive neurons in the lateral septum (C) only poorly co-localizes with MG-160, as demonstrated when fusing images of NT and MG-160 labelling (C'). Similarly, E6I-immureactivity (D) shows minimal overlap with MG-160 immunolabelling in fused images from the same region (D'). E: Dual immunostaining of NT (E) and E6I (E') in the lateral portion of the bed nucleus of the stria terminalis. Note that the two antigens are concentrated within small intracytoplasmic granules. Merging of (E) and (E') demonstrates complete overlap between NT and E6I distributions (E''). Scale bar = 10 μm.


PC5
A
MG-160
A'
PC5/MG-160
A''
PC2
B
MG-160
B'
PC2/MG-160
B''
NT
C
NT/MG-160
C'
E6I
D
E6I/MG-160
D'
NT
E
E6I
E'
NT/E6I
E''

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CONNECTING TEXT # 2

Based on our observations presented in Chapter 3, it seems probable that PC2 and PC5A play a role in the processing of pro-NT/NN since NT co-localized extensively with both proteases in most brain regions examined. However, only small proportions of NT neurons co-expressed either PC2 or PC5A in the nucleus accumbens, striatum, or diagonal band of Broca, suggesting that other proteases are involved in the processing of pro-NT/NN. A potential candidate is PC1, a convertase specific to the regulated pathway of secretion and which is highly expressed in the brain (Schafer et al., 1993; Winsky-Sommerer et al., 2000). PC1 expressed in a number of NT-rich regions including the lateral septum, diagonal band of Broca, medial preoptic area, paraventricular nucleus of the thalamus and central nucleus of the amygdala (Winsky-Sommerer et al., 2000). In addition, *in vitro* experiments performed on stably-transfected endocrine cells co-expressing pro-NT/NN and PC1 showed that this protease processed the NT/NN precursor efficiently (Rovere et al., 1996).

We therefore propose in Chapter 4 to determine the extent of co-localization of PC1 with NT in the rodent brain using a combination of simple and dual immunohistochemistry.

Chapter Four

Immunohistochemical evidence for the implication of PC1 in the processing of pro-neurotensin in rat brain

IMMUNOHISTOCHEMICAL EVIDENCE FOR THE IMPLICATION OF PC1 IN THE PROCESSING OF PRO-NEUROTENSIN IN RAT BRAIN

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Short title: Co-localization of neurotensin with PC1.

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ABSTRACT

Biochemical studies have shown that prohormone convertases PC1, PC2 and PC5A all have the capacity to process, with different specificities, the neurotensin/neuromedin N precursor, pro-NT/NN. A previous study from our laboratory has demonstrated that in rat brain, both PC2 and PC5A may be co-expressed with NT, lending support to a physiological implication of these two enzymes in the endoproteolytic maturation of pro-NT/NN. In the present study, we sought to determine whether PC1 might also be involved in this process by comparing the immunohistochemical distribution of the enzyme with that of NT in both singly labeled and dually labeled serial brain sections. PC1 was found to co-localize extensively with NT throughout the rat neuraxis. However, there were important regional variations in the proportion of NT neurons co-expressing PC1. Furthermore, this proportion was negatively correlated with that of NT neurons expressing PC5, suggesting that PC1 may serve as an alternative to PC5A for processing pro-NT/NN in mammalian brain.

Indexing terms: protein convertases, immunohistochemistry, neuropeptides, propeptides, protease, subtilisin, kexin.

INTRODUCTION

Peptide neurotransmitters are generated by endoproteolytic cleavages of larger precursor molecules. These cleavages usually occur at sites flanked by pairs of basic residues and are mediated by enzymes of the prohormone convertase (PC) family, which include furin, PC1 (also known as PC3), PC2, PC4, PACE4, PC5A/B (also known as PC6), PC7 (also known as PC8 or LPC) and SKI-1 (for review see (Seidah and Chretien, 1999)). Of these, PC1, PC2 and one of the two isoforms of PC5, PC5A, were shown to be sorted through the regulated secretory pathway of secretion (Benjannet et al., 1993) (De Bie et al., 1996) (Barbero et al., 1998) and to be involved in the processing of neuropeptide precursors (see (Seidah and Chretien, 1999) for review). Current evidence suggest that the endoproteolytic specificity of individual PCs rests with both their ability to accommodate putative substrates and their expression within the same cells as these substrates To date, PC1, PC2 and PC5A have been shown to cleave a variety of propeptides *in vitro*, but little is known of their association with these putative substrates *in vivo* (for review see Seidah and Chretien, 1999).

Neurotensin (NT) and Neuromedin N (NN) are two structurally related peptides that share a common peptide precursor, pro-NT/NN (Fig. 1). Both NT and NN are highly expressed in the gut and adrenal medulla, but also in the brain where they serve as neurotransmitters/neuromodulators. The processing of pro-NT/NN is tissue-specific in that the proportion of maturation products (Fig. 1) varies widely between brain (de Nadai et al., 1994; Woulfe et al., 1994), gut (Carraway and Mitra, 1990), and adrenal medulla (Carraway et al., 1993). In addition, within the brain, there are regional differences in the

relative distribution of the various pro-NT/NN maturation products (Kitabgi et al., 1991; de Nadai et al., 1994).

Experiments in neuroendocrine cells endogenously expressing pro-NT/NN and transfected with either PC1, PC2 or PC5A have shown that all of these PCs have the potential to cleave pro-NT/NN, albeit with different specificities. Furthermore, we recently demonstrated that NT co-localized with both PC2 and PC5A in rat brain, thereby providing anatomical evidence for a physiological role of both PC2 and PC5A, either alone or in combination, in pro-NT/NN maturation. However, our immunohistochemical data also suggested that other PCs might be involved in the *in vivo* endoproteolytic maturation of pro-NT/NN. In the present study, we attempted to determine whether PC1 might play such a role, by determining the extent to which it co-localized with NT and how this co-localization complemented that with PC2 and PC5A.

MATERIALS AND METHODS

Antibodies: The PC1 antibody was raised in rabbit against the carboxy-terminal segments 629-726 of mPC1 (Benjannet et al., 1991) (Seidah et al., 1991). Biochemical and immunohistochemical characterisations of this antibody have been reported elsewhere (Benjannet et al., 1993; Marcinkiewicz et al., 1993; Winsky-Sommerer et al., 2000). Antibodies against NT raised in rabbit (Incstar) and rat (Eugene Tech) were used for single- and double-labeling experiments, respectively.

Colchicine administration: All animal procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee. Optimal visualization of NT-immunoreactive cell bodies required pre-treatment of the animals with colchicine. Sprague-Dawley rats (260-280g; Charles-River Canada) were injected stereotaxically with 30 µl of colchicine (2 mg/ml) into the left lateral ventricle. They were fixed 48h later by intra-aortic perfusion of 500 ml of 4 % paraformaldehyde, 0.2 % picric acid in 0.2 M phosphate buffer (PB). Brains were removed, postfixed, cryoprotected overnight and frozen in isopentane, as described previously (Villeneuve et al., 2000).

Single-labeling immunohistochemistry: Brains (n=5) were serially sectioned and pairs of adjacent sections were immunolabeled for NT and PC1, as described elsewhere (Villeneuve et al., 2000). Briefly, sections were blocked with normal goat serum (NGS), incubated overnight at 4 °C with antibodies against NT (series 1; 1:20 000) or PC1 (series 2; 1:800), both in Tris buffer saline (TBS) containing 0.1 % Triton X-100 and 1 %

NGS. On the next day, sections were processed for immunohistochemistry using a biotinylated tyramine-amplified version of the avidin-biotin peroxidase method, as described earlier (Villeneuve et al., 2000). Immunolabeling was revealed by incubating the section with diaminobenzidine-H_2O_2.

Double-labeling immunohistochemistry: Additional sections were processed for dual NT/PC1 immunofluorescence (n=6). After rinsing in TBS, sections were blocked in TBS containing NGS and incubated overnight at 4 °C in a TBS solution containing 1 % NGS, 0.2 % Triton X-100, and a mixture of rat NT (1:1000) and PC1 (1:800) antibodies. NT-labeling was revealed by indirect immufluorescence using an Alexa 488 -tagged goat anti-rat antibody (Molecular Probes), whereas PC1 was visualized using Texas-Red-conjugated streptavidin (Jackson ImmunoResearch) amplified with ABC and biotinylated tyramine, as described elsewhere (Villeneuve et al., 2000). Images were examined with a Zeiss inverted confocal microscope and processed using the Carl Zeiss CLSM software (version 3.1). Colour images were sharpened and adjusted for contrast and brightness using Adobe Photoshop 5.5 software (Adobe).

Specificity controls: Method specificity was assessed in all experiments by omitting the primary antibodies. In addition, the absence of cross-reactivity of the secondary antibodies in double-labeling experiments was assessed by omitting one of both of the primary antibodies during the overnight incubation. Finally, the specificity of the PC1 immunolabeling was assessed by preadsorption of the antibody with its cognate peptide.

Statistics: The distribution of NT neurons co-expressing PC1 was correlated with that previously reported by us for neurons co-expressing NT and PC5A (Villeneuve et al., 2000). NT neurons of the periventricular and paraventricular nuclei of the hypothalamus were excluded from the calculations since NT is not expressed in those regions of naive animals (Kiyama and Emson, 1991). Statistical calculations were performed using the GraphPad Prism program (GraphPad software, version 2.5).

RESULTS

As evident in serial, DAB-revealed sections, colchicine treatment significantly increased the number of NT-immunopositive cell bodies detected in rat brain as compared to non-colchicine treated rats (not shown). By contrast, colchicine treatment altered neither the number nor the labeling density of PC1-immunoreactive cells. Immunohistochemical controls, in which the primary antibodies were omitted or the antiserum was pre-absorbed with the PC1 antigenic peptide, were devoid of immunohistochemical signal.

Throughout the neuraxis, PC1 immunoreactivity was more widely distributed than that of NT. At the cellular level, PC1 immunoreactivity was mainly concentrated within neuronal perikarya but also extended in dendrites. We could not ascertain whether axon terminals were labeled as well. By contrast, NT immunoreactivity was evident in neuronal perikarya, dendrites, and axon terminals.

Single labeling studies: In conformity with earlier studies, regions expressing the highest densities of NT-immunoreactive nerve cell bodies included the nucleus accumbens, lateral septum, caudate/putamen, diagonal band of Broca, bed nucleus of the stria terminalis, preoptic area, central nucleus of the amygdala, paraventricular nucleus of the thalamus, paraventricular, periventricular, lateral and arcuate nuclei of the hypothalamus, ventral tegmental area, periaqueductal gray and dorsal raphe (Table 1). All of these regions also contained a significant number of PC1-positive cells. In the nucleus accumbens, NT-immunolabeled neurons were more numerous than PC1-immunoreactive ones, especially in the shell of the nucleus. In the lateral septum, NT-

immunopositive perikarya were numerous rostral to the decussation of the anterior commissure, after which their number decreased abruptly. Within the same region, PC1-immunoreactive cells were less intensely labeled and less numerous than NT-neurons and were apparent throughout the rostro-caudal extent of the nucleus. In the caudate/putamen, NT-immunoreactive neurons were small, intensely labeled and confined to the dorsomedial portion of the structure, whereas PC1-immunopositive neurons comprised both small and large cells, were only weakly labeled, and were distributed throughout the nucleus. In the diagonal band of Broca, NT-immunolabeled neurons were sparse and moderately immunoreactive whereas PC1-immunoreactive cells were numerous and intensely labeled. In the lateral portion of the bed nucleus of the stria terminalis, there were high densities of both NT- and PC1-immunolabeled neurons (Fig. 2). In the medial preoptic area, NT-immunopositive cells were both numerous and highly immunoreactive. In the same region, PC1-immunoreactive neurons were less intensely labeled but their density was similar to that of NT cells.

In the paraventricular nucleus of the hypothalamus NT-immunopositive neurons were intensely labeled and confined to the parvocellular segment. By contrast, PC1-immunoreactive neurons were only moderately reactive and concentrated within the magnocellular tier. Moderate to small numbers of both NT- and PC1-immunoreactive cells were also detected in the arcuate and periventricular nuclei. The lateral hypothalamus exhibited a large contingent of neurons strongly immunoreactive for NT and an equally large population of neurons strongly immunoreactive for PC1 (Fig. 3).

In the thalamus, numerous intensely reactive NT-positive neurons were apparent throughout the paraventricular nucleus. Within the same nucleus, PC1-immunopositive

neurons were as numerous, but less intensely labeled than NT-labeled ones. More ventrally, large numbers of intensely labeled NT neurons were detected in the central nucleus of the amygdala. Within the same region, PC1 immunopositive neurons were less intensely labeled and slightly less numerous than NT cells.

In the midbrain, NT perikarya were scattered in the ventral tegmental area. PC1 neurons were also present, and in greater number, within this area. More laterally, the substantia nigra pars compacta contained only few NT- and PC1-immunolabeled neurons. The periaqueductal gray contained numerous NT-immunoreactive cells, particularly in the ventral and lateral portions of the structure. Within the same area, PC1 immunoreactive neurons were more numerous and more evenly distributed than NT-immunopositive ones.

Finally, in the dorsal raphe nucleus, numerous strongly NT-immunopositive neurons were evident. PC1-immunolabelled neurons were as intensely immunoreactive, but more numerous than NT cells.

Double labeling studies: To determine whether NT immunoreactive neurons co-expressed PC1, we quantitatively assessed the proportion of NT-immunopositive neurons that were positive for PC1. Texas Red-labeled PC1 and Alexa 488-labelled NT showed essentially the same topographic distribution as the DAB–revealed antigens in single labeling experiments. PC1 co-localized with NT in all regions examined, although regional variations were evident (Table 1). Thus, the highest proportion of NT neurons co-localizing PC1 was detected in the central nucleus of the amygdala (Fig. 4). High proportions of NT-immunopositive neurons co-expressing PC1 were also found in the

lateral portion of the bed nucleus of the stria terminalis (BNL) and in the lateral hypothalamus (LH; Fig. 5). The proportion of NT-immunopositive neurons co-localizing PC1 was moderate in the nucleus accumbens, lateral septum (LS), medial preoptic area (MPA), arcuate nucleus of the hypothalamus, periaqueductal gray (PAG) and dorsal raphe (DR). Finally, only few neurons were dually labeled for NT and PC1 in the paraventricular and periventricular nuclei of the hypothalamus and in the ventral tegmental area.

TABLE, FIGURES AND FIGURE LEGENDS

Table 1: Co-localization of NT and PC1 in rat brain

Region	NT*	PC1*	(NT-PC1)/NT$^{\Psi}$ (% total NT cells)
Nucleus accumbens	+++	+	43
Lateral septum	+++	++	45
Caudate/putamen	+	+++	73
Diagonal band of Broca	+	+++	ND
Bed nucleus of the stria terminalis, lateral	++++	+++	61
Bed nucleus of the stria terminalis, ventral	+++	+/-	ND
Medial preoptic area	+++	+++	39
Lateral preoptic area	+/-	+	ND
Paraventricular nucleus, hypothalamus	+++	++++	24
Periventricular, hypothalamus	+/-	+	33
Arcuate nucleus, hypothalamus	++	+	38
Lateral hypothalamus	++	++	63
Paraventricular nucleus, thalamus	+++	++	70
Central amygdala	++++	++	76
Ventral tegmental area	+	++	32
Substantia nigra, pars compacta	+	+	ND
Periaqueductal grey	+++	++	55
Dorsal raphe	++	+++	37

* Semiquantitative estimates of the number of NT and PC1 immunoreactive cells: -,not detectable; +/-, very low; +, low; ++, moderate; +++, high; ++++, very high.

$^{\Psi}$ These numbers correspond to the proportion NT neurons dually labeled for PC1 (NT-PC1) over the total number of NT neurons.

Fig. 1. Diagrammatic representation of rat pro-NT/NN and of its major processing products in brain. The propeptide may be cleaved at any of the four Lys-Arg dibasic sites indicated, however, PC1 is reportedly most efficient at the two C-terminal most dibasic residues (modified from Villeneuve et al., 2000 (Villeneuve et al., 2000)).

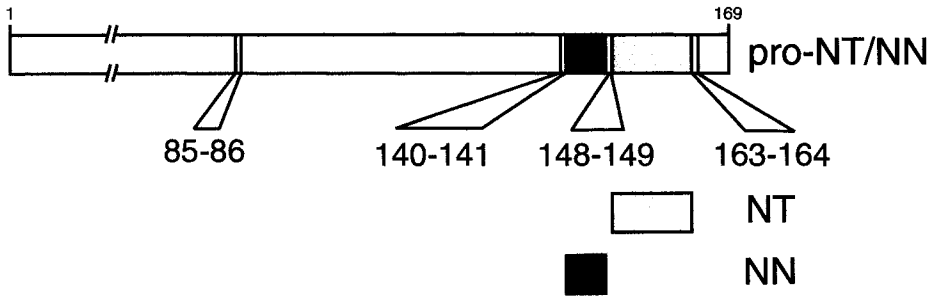

1
169
pro-NT/NN
85-86
140-141
148-149
163-164
NT
NN

Fig. 2. Distribution of PC1- and NT-immunoreactive neurons in adjacent sections from the bed nucleus of the stria terminalis. The two populations of immunolabeled cells overlap extensively in the lateral portion of the bed nucleus of the stria terminalis (BNL). ic, internal capsule; BNM, bed nucleus of the stria terminalis, medial anterior. Scale bar = 130 μm.

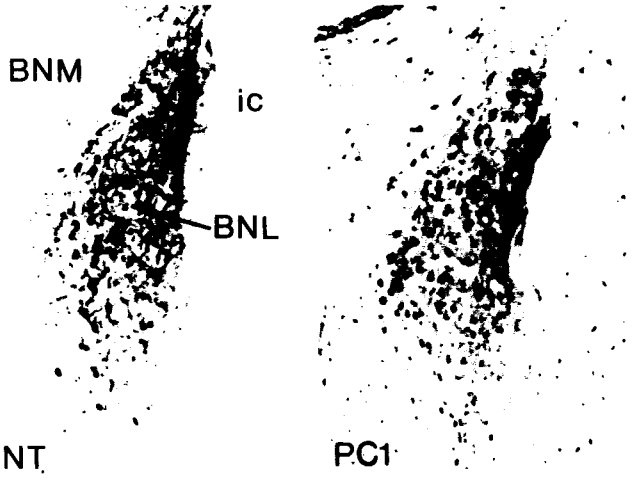

BNM
ic
BNL
NT
PC1

Fig. 3. Distribution of PC1- and NT-immunoreactive neurons in adjacent sections from the lateral hypothalamus (LH). Both populations are co-extensive in LH. However, whereas NT positive cells are restricted to this region, PC1 immunolabeled neurons extend to surrounding structures. ot, optic tract; ic, internal capsule. Scale bar = 320 μm.

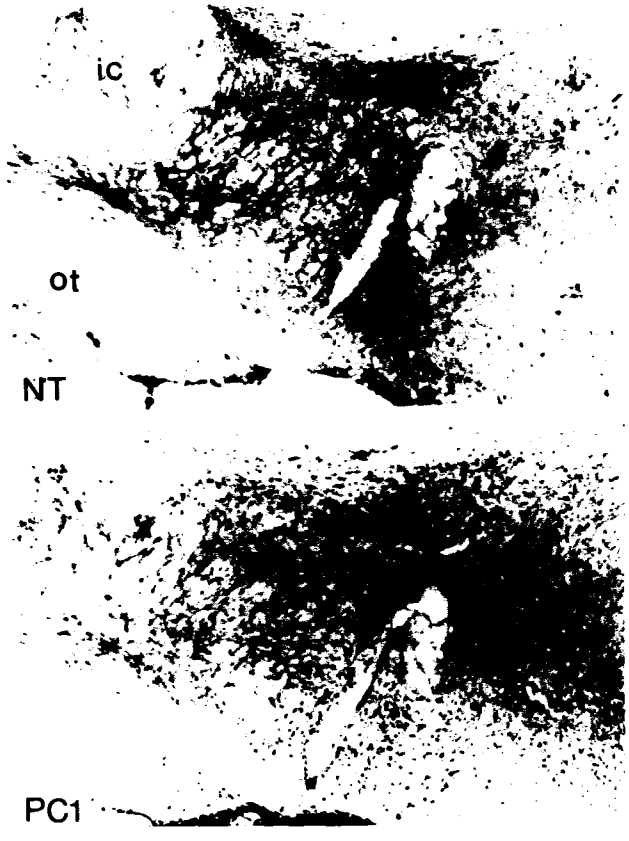

ic
ot
NT
PC1

Fig. 4. Dual PC1- (A, B) and NT- (A', B') immunolabeling in the nucleus of the central amygdala (A, A') and in the lateral hypothalamus (B, B'). Single confocal microscopic images scanned sequentially for FITC (A,B) and Texas red (A',B') and averaged over 32 scans per frame. In the central nucleus of the amygdala, most NT-immunolabeled neurons are dually labeled for PC1 (arrows). Note that only a few NT-immunopositive neurons are negative for PC1 (stars) whereas singly labeled PC1 neurons are more numerous. In the lateral hypothalamus, a high proportion of NT neurons co-localize PC1 (arrows). Again, a few NT-immunolabeled neurons do not co-express PC1 (stars). Scale bar = 20 μm.

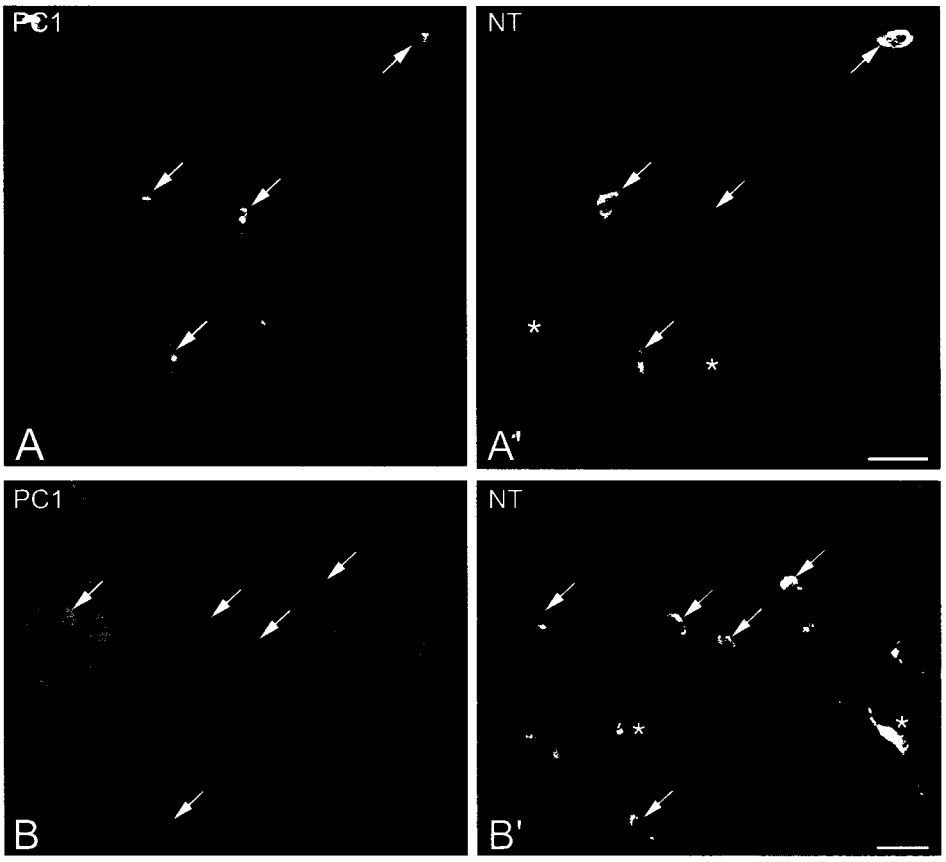
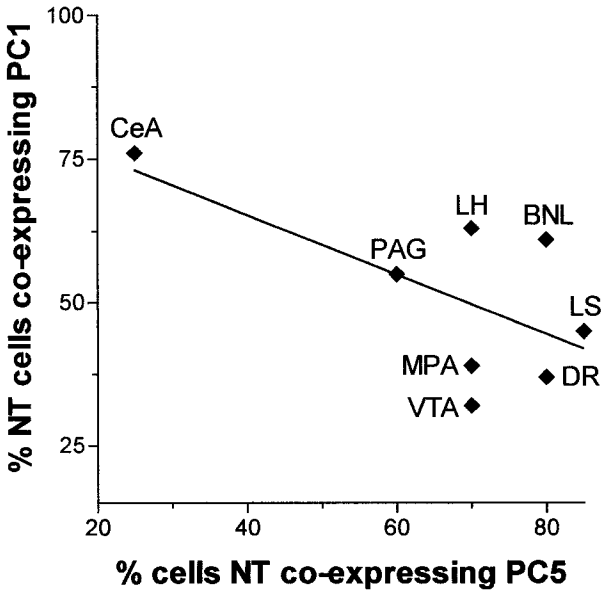

PC1
NT
A
A'
PC1
NT
B
B'

Fig. 5. Correlation between the proportions of NT neurons co-localizing PC1 and PC5A in a number of brain regions ($r = -0.64$, $df = 7$; $p = 0.0427$). BNL, lateral portion of the bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; Dr, dorsal raphe; LH, lateral hypothalamus; LS, lateral septum; MPA, medial preoptic area; PAG, periaqueductal grey; VTA, ventral tegmental area.


100
75
50
25
% NT cells co-expressing PC1
CeA
LH
BNL
PAG
LS
MPA
DR
VTA
20
40
60
80
% cells NT co-expressing PC5

DISCUSSION

The present study demonstrates extensive cellular co-localization between the neuropeptide neurotensin (NT) and the pro-hormone convertase PC1 in the rat central nervous system and thereby provides anatomical evidence for a physiological role of PC1 in the maturation of pro-NT/NN in mammalian brain.

The specificity of the C-terminus-directed mPC1 antibody used in the present experiments has been extensively discussed elsewhere (Benjannet et al., 1993; Marcinkiewicz et al., 1993; Winsky-Sommerer et al., 2000). This antibody was shown to recognize the enzymatically active 87 kDa form of PC1 in the adult rat brain, but not its shorter, 66 kDa C-terminally-truncated derivative (Dong et al., 1997; Winsky-Sommerer et al., 2000). However, studies using N-terminal-directed mPC1 antibodies showed the 66 kDa product to be systematically present within the same cells as the 87 kDa form (Vindrola and Lindberg, 1992) (Rovere et al., 1996; Winsky-Sommerer et al., 2000), suggesting that the distribution of the 87 kDa-immunoreactive cells reported here reflects that of PC1-expressing cells in rat brain. Further evidence for the specificity of our PC1 immunohistochemical signal includes: (1) absence of immunostaining following preadsorption of the immune serum with the cognate peptide; (2) congruence between the distribution of immunolabeled cells and that of PC1-expressing neurons previously documented by immunohistochemistry (de Nadai et al., 1994; Winsky-Sommerer et al., 2000) or *in situ* hybridization (Schafer et al., 1993).

The specificity of the NT antibody used in the present work has also been extensively verified elsewhere (Bayer et al., 1991). Accordingly, the distribution of NT-

immunoreactive cells observed here closely conformed to that reported earlier in the brain of colchicine-treated rats (Jennes et al., 1982) (de Nadai et al., 1994).

At the light microscopic level, PC1-immunoreactive neurons were detected in all areas containing NT-immunoreactive nerve cell bodies. They also were evident in several additional brain regions, in keeping with the implication of this protein convertase in the processing of a wide variety of propeptides (for review see Seidah and Chretien, 1999). Within NT-containing areas, there was no direct correlation between the density of PC1-immunoreactive cells and that of NT-positive neurons confirming that PC1 did not preferentially target pro-NT/NN. At the confocal microscopic level, however, there was unequivocal expression of immunoreactive PC1 in a substantial sub-population of NT-labeled cells. This observation, together with biochemical data demonstrating efficient *in vitro* processing of pro-NT/NN by PC1 (Rovere et al., 1996), suggest that PC1 is implicated in the processing of pro-NT/NN in central neurons.

In a previous study, we provided immunohistochemical evidence for the involvement of both PC2 and PC5A in the maturation of pro-NT/NN in central neurons (Villeneuve et al., 2000). However, whereas a high proportion of NT-positive neurons co-localized PC2 throughout the brain, the proportion of NT-immunoreactive cells expressing PC5A displayed much greater regional variation (Villeneuve et al., 2000). NT neurons co-localizing PC1 also showed marked regional differences, but such that their distribution was negatively correlated with that of NT neurons co-localizing PC5A. Thus, the highest proportion of NT neurons dually positive for PC1 was found in the central nucleus of the amygdala, a region where the lowest proportion of NT cells positive for PC5A had previously been observed (Villeneuve et al., 2000). Conversely, the bed nucleus of the

stria terminalis, ventral tegmental area, and nucleus raphe dorsalis contained only few NT cells dually positive for PC1, but high proportions of NT neurons immunopositive for PC5A. Such negative correlation might reflect a functional complementarity between PC1 and PC5A within NT cells.

Biochemical and cell biological studies have shown PC1 and PC5A to share functional features distinct from those of PC2. First, both were found to be present in enzymatically active form as proximally as the Golgi apparatus within the secretory pathway (Benjannet et al., 1992; Benjannet et al., 1993; Hornby et al., 1993; De Bie et al., 1996; Barbero et al., 1998; Villeneuve et al., 2000). By contrast, PC2-mediated processing was reported to take place more distally, partly in the Trans-Golgi Network but mainly in secretory granules (Benjannet et al., 1992; Shen et al., 1993) (Benjannet et al., 1993). Second, PC1 and PC5A were both shown *in vitro* to preferentially cleave the last C-terminal doublets of pro-NT/NN (Lys^{163}-Arg^{164} and Lys^{148}-Arg^{149}), whereas PC2 preferentially cleaves Lys^{140}-Arg^{141} and Lys^{148}-Arg^{149} sites (Rovere et al., 1996) (Barbero et al., 1998). In light of these earlier findings, the present results suggest that PC1 and PC5A might be alternately complementing PC2 to optimize the cleavage of the three terminal doublets of the pro-NT/NN precursor and thereby provide for efficient processing of the short forms of NT and NN prevalent in the brain (de Nadai et al., 1994). The relative proportion of PC1, PC2 and/or PC5A expressed by individual cells would ultimately chisel the final peptide output and account for the regional variations in NT/NN ratios reported in mammalian central nervous system (de Nadai et al., 1994).

In conclusion, the present report provides the first evidence for the presence of the prohormone convertase PC1 in central NT neurons and suggests that this convertase may

serve as an alternative to PC5A to process, in conjunction or not with PC2, pro-NT/NN in mammalian brain.

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CONNECTING TEXT # 3

Although our work provides evidence for a role of PC1, PC2 and PC5A in the physiological maturation of pro-NT/NN, such a role still remains to be confirmed and better characterized. In addition, the possible role of PC7 in the processing of pro-NT/NN has never been investigated. In the next series of experiments presented in Chapter 5, we propose to determine the pro-NT/NN maturation profiles in brains of mice invalidated for either PC7 (PC7-/-), PC2 (PC2-/-), and/or PC1 (PC1+/- and PC2-/-; PC1+/-). Pro-NT/NN maturation products will be determined by a combination of radioimmunoassay and immunohistochemical techniques. Comparison of the pro-NT/NN maturation profiles between WT and the KO mice will allow us to determine the relative contribution of PC1, PC2 and PC7 in the processing of pro-NT/NN in the rat brain.

Chapter Five

Altered processing of the neurotensin/neuromedin N precursor in PC2 knock down mice: A biochemical and immunohistochemical study

ALTERED PROCESSING OF THE NEUROTENSIN/NEUROMEDIN N PRECURSOR IN PC2 KNOCK DOWN MICE: A BIOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY

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Short title: Defective Pro-NT/NN processing in PC2 null mice.

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Abbreviations used: NT, neurotensin; NN, neuromedin N; pro-NT/NN, pro-neurotensin/neuromedin N; PC, prohormone convertase; KO, knock out, RIA, radioimmunoassay; iNT, immunoreactive NT; iNN, immunoreactive NN; iK6L, immunoreactive K6L; CTiNN, citraconylated, trypsin-digested iNN; HPLC, high pressure liquid chromatography.

ABSTRACT

Neurotensin (NT) and neuromedin N (NN) are generated by endoproteolytic cleavage of a common precursor molecule, pro-NT/NN. To gain insight into the role of prohormone convertases PC1, PC2, and PC7 in this process, we investigated the maturation of pro-NT/NN in the brain PC7 (PC7-/-), PC2 (PC2-/-), and/or PC1 (PC1+/- and PC2-/-; PC1+/-) knock down mice. Inactivation of the PC7 gene was without effect, suggesting that this convertase is not involved in the processing of pro-NT/NN. By contrast, there was a 15 % decrease in NT and a 50 % decrease in NN levels, as measured by radioimmunoassay, in whole brains extracts from PC2 null as compared to wild type mice. Using immunohistochemistry, we found that this decrease in pro-NT/NN maturation products was uneven and that it was most pronounced in the medial preoptic area, lateral hypothalamus, and paraventricular hypothalamic nuclei. These results suggest that PC2 plays a critical role in the processing of pro-NT/NN in mouse brain and that its deficiency may be compensated to a regionally variable extent by other convertases. Previous data have suggested that PC1 might be subserving this role. However, there was no change in the maturation of pro-NT/NN in the brain of mice in which the PC1 gene had been partially inactivated, implying that complete PC1 knock down may be required for loss of function.

INTRODUCTION

Neurotensin (NT) and neuromedin N (NN) are two structurally related peptides that are processed from a common 169-170 amino-acid precursor molecule, pro-NT/NN (Dobner et al., 1987; Kislauskis et al., 1988). Both NT and NN are expressed in the brain and gut where they act as neurotransmitter/neuromodulators and local hormones, respectively (Rostene and Alexander, 1997). In addition to NT and NN, pro-NT/NN contains two other potentially bioactive peptides, namely large NT and large NN.

The structure of the NT/NN precursor molecule is schematically represented in Fig. 1. NT is located in the C-terminal region of the precursor tail and is preceded by NN. The two peptides are separated by a pair of basic residues (Lys^{148}-Arg^{149}). Two other Lys-Arg sequences (Lys^{140}-Arg^{141} and Lys^{163}-Arg^{164}) flank the N-terminus of NN and the C-terminus of NT, respectively. An additional Lys-Arg site is located at positions 85-86 of the precursor. Cleavage at these doublets will lead to the release of the mature products from the precursor molecule in a tissue-specific manner. Thus, in the gut, pro-NT/NN is predominantly cleaved at the level of the last two basic residues, resulting into the production of NT and large NN and, to a lesser extent, of NN (Carraway and Mitra, 1990; Shaw et al., 1990). By contrast, in the brain, the last three pairs of basic residues are cleaved almost to the same extent, giving rise to high amounts of NT and NN and to lesser amounts of large NN (Kitabgi et al., 1991; de Nadai et al., 1994). Furthermore, in the brain, the processing of pro-NT/NN is region-specific in that the relative proportions of NT and NN vary among brain structures (Kitabgi et al., 1991) and that cleavage at the Lys^{85}-Arg^{86} residues only occurs in selective brain regions (de Nadai et al., 1994; Woulfe et al., 1994).

The endoproteolytic processing of peptide precursors at pairs of basic residues is mediated by enzyme members of the prohormone convertase (PC) family. Seven such enzymes have so far been identified. They are referred to as PC1/PC3 (EC 3.4.21.93), PC2 (EC 3.4.21.94), furin (EC 3.4.21.75), PACE4, PC4, PC5/PC6, and PC7/LPC/PC8/SPC7 (Seidah and Chretien, 1999; Bergeron et al., 2000). Of these, PC1, PC2, and PC5A have been shown to specifically process peptide precursors routed to the regulated secretory pathway (Lindberg et al., 1994; Malide et al., 1995; De Bie et al., 1996). *In vitro* studies have shown that these three enzymes differentially process pro-NT/NN, giving rise to distinct combinations of processing products (Rovere et al., 1996; Barbero et al., 1998). All three convertases co-localize extensively with NT in rat brain, suggesting that they may also be involved in the processing of pro-NT/NN *in vivo*. Thus, immunoreactive PC2 is detectable within 50-60 % of central NT neurons (Villeneuve et al., 2000b). PC1 and PC5A co-localize with NT less extensively but in a complementary fashion, in that regions containing the highest number of NT/PC1 dually labelled cells show the smallest number of NT-neurons co-localizing PC5A and vice-versa (Villeneuve et al., 2000a; Villeneuve et al., 2000b).

However, the complex and often overlapping patterns of co-localization of NT with these three PCs make it difficult to assess the relative contribution of these enzymes to the region-specific processing of pro-NT/NN in the brain. In order to address this question, we compared the pattern of pro-NT/NN processing in the brain of mice in which PC2 or PC1 genes had been totally or partially inactivated, respectively, to that in the brain of wild type (WT) mice. PC2 null mice that are heterozygous for PC1 were also studied. Using a battery of antisera directed toward different epitopes of pro-NT/NN, the

nature and concentration of precursor-derived maturation products were determined in whole brain homogenates by radioimmunoassay (RIA) coupled with high pressure liquid chromatography (HPLC), and the regional brain distribution of processing products was investigated by immunohistochemistry.

EXPERIMENTAL PROCEDURES

Animals

WT (n= 25 for immunohistochemistry and n= 9 for radioimmunoassay) and mutant mice (age-matched, 2.5-5 months) were littermates obtained from appropriate mating of WT or mutant C57BL/J mice. PC2 +/- (or -/-) mice were initially provided by Dr. D. F. Steiner (Howard Hughes Medical Institute, University of Chicago, Chicago, IL). PC2-/- mice were generated by introducing a neomycin cassette into exon 3 of the PC2 gene (Furuta et al., 1997). This insertion alters transcript splicing and prevents activation of the mutated PC2 precursor. PC2 -/- (n=8 for immunohistochemistry and n=4 for radioimmunoassay), PC1 +/- (n=6), and PC2-/-; PC1+/- (n=6) mice were developed by M. Mbikay. These mice (n=3) were generated by deletion of exons 3 to 7 from the PC7 gene. PC7 -/- mice were kindly provided by Dr. D. Constan and E. Robertson (Harvard University) and bred in the laboratory of N. G. Seidah (IRCM). All animal procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Synthetic peptides and antisera

The peptides NT, NN, KLPLVL (K6L), and E6I (EKEEVI) were custom synthesized by Neosystem (Strasbourg, France).

The preparation and characterization of the antisera directed against the C-terminus of NT (29I), the N-terminus of NT (28H; gift from Jean-Claude Cuber, Lyon, France), neuromedin N (NN-Ah), K6L (K6L-Af), and E6I (E6I-Ah; see Fig. 1) have been previously described (Bidard et al., 1993). All were shown to preferentially recognize

their respective antigen provided that the adjacent pair of basic residues in the neurotensin/neuromedin N precursor had been cleaved. Thus, the 29I and 28H anti-NT antisera are specific for the intact free C- and N-terminus of NT, exposed after endoproteolytic cleavage at residues Lys^{163}-Arg^{164} and Lys^{148}-Arg^{149}, respectively (see Fig. 1). Similarly, the anti-NN (NN-Ah) and anti-K6L (K6L-Af) antisera are specific for the intact free N-terminus of NN and K6L, exposed after cleavage at residues Lys^{140}-Arg^{141} and Lys^{85}-Arg^{86}, respectively (see Fig. 1; Bidard et al., 1993). The anti-E6I antiserum is specific for the intact free C-terminus of E6I, exposed after cleavage at residues Lys^{140}-Arg^{141}. The NT rabbit antibody used for immunohistochemistry is a polyclonal antibody raised against the whole sequence of NT (Incstar; Stillwater, Mn). The PC1 antiserum was generated against the carboxyterminal segments 629-726 of mPC1 (Benjannet et al., 1991; Seidah et al., 1991). Biochemical and immunohistochemical characterization of the antibody has been reported elsewhere (Dong et al., 1997; Winsky-Sommerer et al., 2000). Finally, the polyclonal antibody directed against PC5A was a generous gift from Dr. A. Franzusoff (University of Colorado Health Sciences Center; Denver, USA).

Tissue extraction

WT and mutant mice were decapitated and the brains were rapidly removed on ice. The brains were then individually weighted and immersed in 10 vol (v/wt) ice-cold 0.1 M HCL and homogenized with a polytron (Brinkmann Instruments, Westbury, NY; 4 times, 10s each time). Extracts were centrifuged at 20 000g for 30 min at 4°C and the supernatants were heated for 10 min in boiling water. For high pressure liquid

chromatography (HPLC), pH was adjusted to 7.2 –7.6 with 0.1 N NaOH and the precipitates were removed by centrifugation (20 000 g, 30 min at 4°C). Extracts were then kept at -20°C for later use. The protein contents of the extracts were determined using the Bio-Rad protein assay reagent according to the procedure recommended by the manufacturer (Bio-Rad laboratories, Richmond, CA).

Radioimmunoassay

Radioimmunoassay conditions were the same as previously described (Bidard et al., 1993). All RIAs were performed in phosphate-buffered saline (PBS; 50 mM Na_2HPO_4 and 140 mM NaCl, pH 7.4) containing 0.1 % gelatin and 0.01 % Triton X-100. Tubes for RIA containing various dilutions of synthetic peptides or unknown samples were incubated for 16-24 h at 4 °C in a final volume of 500 µl containing 7000-8000 c.p.m. of tracer. Separation of bound from free peptide was performed with charcoal (Carraway and Leeman, 1976).

Reverse phase HPLC of tissue extracts

Reverse phase HPLC was performed on supernatants obtained from both WT and mutant animals in order to confirm the nature of the antigens detected by RIA. Brain extracts from both WT and control mice (≅10.8 mg) were injected onto a 4.6 X 250 mm Waters symmetric 300 (C18, 5 µm) column. Elution was carried out in 0.1 % trifluoroacetic acid / 0.05 % triethylamine with a 10 – 40 % linear gradient of acetonitrile over 42 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected, lyophilized and reconstituted in either 1 ml PBS for direct RIA, or in 500 µl 0.05 % Triton X-100 for

trypsinization (see below). The fractions were then assayed for their content in: 1) immunoreactive NT (iNT), N-terminal (detected by the antisera 28H); 2) iNT, C-terminal (detected by the antisera 29I); 3) iNN, as detected with the antisera 29-Ah. Synthetic NT and NN were used to calibrate the column and eluted with retention times of 34-35 and 36-37 min, respectively.

Citraconylation, trypsin digestion and unblocking procedure

Direct RIA only allows the detection of antigenic sites that have been exposed after cleavage of the adjacent pair of basic residues. In order to measure the amounts of unprocessed pairs of basic residues, internal antigenic sites have to be unmasked by trypsin cleavage. Since NN starts with a lysine residue that is essential for antibody recognition, the amine function of the lysine side-chain was reversibly protected by treatment with citraconic anhydride (Wilkinson, 1986). Aliquots from HPLC fractions reconstituted in 0.05 % Triton X-100 or samples not subjected to HPLC were then digested with trypsin to cleave the citraconylated peptide specifically at the C-terminal sides of arginine residues. The extracts were then essayed for iNN. The value of CTiNN thus obtained provides an index of total amount of pro-NT/NN (either processed or unprocessed) that was originally present in the extracts (Bidard et al., 1993).

Immunohistochemistry

Mice were deeply anaesthetized with sodium pentobarbital (80 mg/kg i.p.; Somnotol, MCT Pharmaceuticals, Cambridge, Canada) and fixed by transaortic perfusion of aldehydes. The animals (n = 8 PC2-/- and n = 25 WT) were perfused with 300 ml of a

mixture of 4 % paraformaldehyde and 0.2 % picric acid in 0.1 M phosphate buffer (PB), pH 7.4, at room temperature at a flow rate of 35 ml/min. Brains were dissected out of the skull and post-fixed for 40 min by immersion in the same fixative. Fixed brains were cryoprotected for 24 hours by immersion in a 30 % sucrose solution in 0.2 M phosphate buffer (PB), pH 7.4 at 4 °C and snap-frozen in isopentane at -50 °C. Coronal sections (25-μm-thick) were cut on a freezing microtome and collected in PB containing 0.1 % sodium azide and either used immediately or stored for up to 6 weeks at 4 °C.

Sections from mutant and control animals corresponding to the same anatomical plane were selected at intervals of 100 μm for NT, E6I, and K6L immunohistochemistry, or 200 μm for PC1 and PC5A immunohistochemistry. Briefly, after two rinses in 0.1 M Tris buffer saline, pH 7.4 (TBS), sections were treated for 20 min with 0.3 % H_2O_2 in TBS to quench endogenous peroxidase. They were then preincubated for 30 min in TBS containing 3 % normal goat serum (NGS) and incubated overnight at 4 °C in a TBS solution containing 1 % NGS, 0.2 % Triton X-100 and either NT (1:20 000), E6I (1:6000), K6L (1:6000), PC1 (1:600), PC2 (1:800), or PC5A (1:2000) antibodies. Sections were then rinsed in TBS containing 1 % NGS and incubated for 50 min at room temperature in biotinylated goat anti-rabbit immunoglobulins (1/100; Jackson ImmunoResearch, West Grove, PA). They were then incubated for 50 min in avidin-biotin peroxidase complex (Vector ABC; Vector Laboratories, Burlingame, CA) and subsequently incubated for 10 min in a solution containing 0.1 % biotinylated tyramine (NEN-Dupont, Boston, MA) activated with 0.01 % H_2O_2 for 10 min and reincubated for 50 min in ABC. Visualization of the bound peroxidase was achieved by reaction in a solution of 0.01 M Tris buffer, pH 7.6, containing 0.05 % 3,3' diaminobenzidine, 0.04 %

nickel chloride and 0.01 % H_2O_2. Sections were mounted on gelatin-coated slides, dehydrated in graded ethanols, delipidated in xylene, coverslipped with Permount and examined under a Leitz Aristoplan microscope.

In all experiments, the absence of cross-reactivity of the secondary antibodies was verified by omitting one or both of the primary antibodies during the overnight incubation. In the case of experiments involving immunostaining with PC5A or PC1 antibodies, specificity controls included preadsorbing the antibodies with their cognate immunogenic peptide.

RESULTS

Radioimmunoassays

To ascertain the physiological role of PC1, PC2, and PC7 in the endoproteolytic processing of pro-NT/NN in rodent brain, levels of N- and C-terminal iNT, N-terminal iNN, iK6L, and CTiNN were quantified by radioimmunoassay in whole brain extracts from WT and mutant (PC2-/-; PC1+/-; PC2-/- PC1+/- and PC7-/-) mice. The data are summarized in Table 1.

CTiNN values were obtained using the NN antiserum (directed against the exposed N-terminal portion of NN) on whole brain extracts that had been subjected to trypsin digestion. This procedure allowed for the detection of both free NN and of iNN that was contained within larger NT/NN-precursor-derived polypeptides, thereby providing an indirect measurement of total pro-NT/NN levels. CTiNN values were similar in all experimental models (Table 1), indicating that none of the PC gene inactivations studied here affected the global levels of pro-NT/NN protein in mouse brain.

Brain extracts from WT mice contained similar amounts of C-terminal and N-terminal iNT (Table 1). These levels were comparable in turn to those of CTiNN (Table 1). Concentrations of iNN and iK6L amounted to approximately 65 % and 6 % of CTiNN levels, respectively (Table 1). Partial inactivation of PC1 (PC1+/-) and total inactivation of PC7 (PC7-/-) had no influence on the concentration of all pro-NT/NN antigens assayed (Table 1). Therefore, the partial or total inactivation of PC1 or PC7 genes, respectively, had no consequence on the processing pattern of pro-NT/NN in mouse brain.

In sharp contrast, total inactivation of the PC2 gene produced marked changes in the concentrations of pro-NT/NN-derived immunoreactive products. Thus, although N-terminal iNT concentrations were similar in brain extracts from PC2-/- and WT mice, C-terminal iNT contents were reduced by almost 20 % in PC2-/- mouse brain as compared to WT (Table 1). Even more drastic was the reduction (approximately 50 %) in the levels of iNN and iK6L in brain extracts from PC2-/- as compared to WT animals (Table 1). Concentrations of immunoreactive products detected in the brain of PC2-/-; PC1+/- mice were not significantly different from those measured in PC2-/- animals, indicating that partial inactivation of PC1 had no major effect on pro-NT/NN processing in PC2 null mice.

Whole brain extracts from all animal models were also subjected to reverse phase HPLC in order to confirm the nature of the immunoreactive products. The purified fractions were either directly assayed for iNT (using N- and C-terminal-directed antibodies) and for iNN, or indirectly assayed for CTiNN following trypsin digestion (Fig. 2). The HPLC profile obtained with brain extracts from WT mice is illustrated in Fig. 2A. A prominent iNT peak eluted at 34-35 min. This peak was detected equally well with N- and C-terminal-directed antibodies and coeluted with synthetic NT, indicating that it corresponded to authentic NT. A minor iNT peak eluting with a retention time of 31-32 min was detected using the N-terminal, but not the C-terminal NT antiserum (Fig. 2A). This material could correspond either to a C-terminally-truncated or to an extended form of NT. Since C-terminally extended NT has previously been shown to elute earlier than NT because of its polar amino-acid content (KRGSYYY; Bidard et al., 1993), the material detected at 31-32 min more likely corresponds to a C-terminally extended form

of the peptide. From now on, this form will be referred to as N-terminal NT. An iNN peak eluted with a retention time of 35-36 min (Fig. 2A). Synthetic NN eluted with the same retention time as this material, suggesting that it corresponded to authentic NN. Finally, CTiNN-containing fractions eluting at 53-56 min were detected (Fig. 2A). The fact that neither N-terminal nor C-terminal iNT were present in these fractions suggested that they consisted of large NN and were devoid of large NT.

Overall, the HPLC profiles obtained from brain extracts of PC1+/- and PC7-/- mice did not differ from those of WT animals (Fig. 2, A-C). In contrast, the profiles from PC2-/- and PC2-/-; PC1+/- mouse brain extracts (Fig. 2, D and E) were clearly distinct from those of WT, PC1+/- and PC7-/- animals. The differences were a marked decrease in the size of the NN peak, a decrease in the size of the NT peak, and an increase in the sizes of large NN and N-terminal NT peaks in PC2-/- and PC2-/-; PC1+/- as compared to PC1+/- and PC7-/- brain extracts.

Based on this HPLC identification, the results of our radioimmunoassays could then be expressed as percentages of pro-NT/NN concentrations by equating C-terminal iNT to NT, iNN to NN, [CTiNN minus iNN] to large NN, [N-terminal iNT minus C-terminal iNT] to N-terminal NT, iK6L to K6L, and CTiNN to pro-NT/NN. As illustrated in Fig. 3, brain extracts from WT mice contained higher levels of NT than of NN. They contained only low concentrations of large NN and even lower amounts of K6L (Fig. 3). N-terminal NT was barely detectable and large NT was undetectable (Fig. 3). The patterns of NT/NN maturation observed in PC1+/- (Fig. 3) and PC7-/- (not shown) mice were very similar to that of WT animals. In contrast, in PC2-/- and PC2-/-;PC1+/- animals, there was a drastic decrease (60 %) in NN levels and compensatory increase in

large NN (Fig. 3). There was also a substantial decrease in the levels of K6L in PC2-/- and PC2-/-;PC1+/- as compared to WT mice (Fig. 3). Finally, there was a significant decrease (20-25 %) in the concentration of mature NT and a concomitant increase in that of N-terminal NT as compared to WT animals.

The percentage of cleavage at the pairs of basic residues in the NT/NN precursor was then calculated by dividing the values of mature products by the total initial values of the NT/NN precursor as determined by CTiNN measurements. As can be seen in Fig. 4, the percentage of cleavage at each site showed little variation between WT and PC1+/- and PC7-/- mice. By contrast, the Lys^{163}-Arg^{164} residues, which were extensively processed in WT animals, were less efficiently cleaved in both PC2-/- and PC2-/-; PC1+/- animals (Fig. 4). The Lys^{148}-Arg^{149} doublet was fully processed in both WT and PC2 null mice (Fig. 4). The Lys^{140}-Arg^{141} was efficiently processed in WT animals but markedly less so in animals inactivated for PC2 (Fig. 4). Finally, the Lys^{85}-Arg^{86} doublet was only poorly processed in WT animals, but even less efficiently cleaved in PC2 knocked down mice (Fig. 4).

Immunohistochemical distribution of NT, E6I and K6L immunoreactivity in normal and PC2-/- mice brain

To determine whether the defects in pro-NT/NN maturation detected by RIA in whole brain extracts from PC2-/- animals were similar in all pro-NT/NN-expressing regions, the immunohistochemical distribution of NT, of the exposed C-terminal extremity of the Glu^{134}-Val^{135}-Ile^{136} sequence (E6I), and of the exposed N-terminal

extremity of the Lys^{87}-Leu^{92} sequence (K6L) of pro-NT/NN were compared between WT and PC2-/- mice.

In both WT and mutant mice, immunoreactivity for NT, E6I, and K6L was almost exclusively confined to axons and axonal varicosities (Figs. 5-7). The topographical distribution of these immunoreactive axons varied markedly between brain regions and generally conformed to the reported distribution of NT immunoreactivity in rodent brain (Woulfe et al., 1994). NT and E6I arborizations were very similar to one another, both in terms of distribution and of labelling intensity. By contrast, K6L-immunoreactive axons exhibited a more restrictive distribution and, overall, gave rise to a weaker immunohistochemical signal. They were, however, detected in most NT- and E6I-containing regions. Only a few NT- or E6I-immunoreactive nerve cell bodies were detected in these non colchicine-treated brains (Figs 5-7). K6L-immunoreactive perikarya were even less numerous and detected only in the ventral portion of the bed nucleus of the stria terminalis and the central nucleus of the amygdala (Fig. 5).

In most areas enriched in NT/E6I/K6L immunoreactivity, there was little detectable difference in either the distribution or the intensity of immunoreactivity for all 3 antigens between WT and PC2 KO animals. Such was the case, for instance, of immunoreactive fiber networks detected in the nucleus accumbens, bed nucleus of the stria terminalis, lateral septum, diagonal band of Broca, caudate-putamen, central nucleus of the amygdala (Fig. 5), paraventricular nucleus of the thalamus, ventral tegmental area, substantia nigra pars compacta, and dorsal raphe nucleus.

By contrast, in the medial preoptic area (Fig. 6), lateral hypothalamic area (Fig. 7), and paraventricular hypothalamic nucleus (not shown), there was a marked decrease in

the density of NT-, E6I-, and K6L-immunoreactive axons in PC2 null as compared to WT mice. A similar decrease in immunostaining density was detected in the arcuate nucleus of the hypothalamus for NT and E6I immunoreactivity, but not for K6L.

Immunohistochemical distribution of PC1 and PC5A in WT and PC2 null mice

To determine whether the introduction of an enzymatically inactive PC2 gene led to region-specific up- or down-regulation of PC1 and PC5A, we compared the immunohistochemical distribution of these two convertases in WT vs PC2-/- animals. Both antigens exhibited a widespread brain distribution consistent with earlier reports (Villeneuve et al., 1999; Winsky-Sommerer et al., 2000). Neither the distribution nor the intensity of either PC1 (Fig 8) or PC5A- (Fig. 9) immunoreactive signals were altered following inactivation of the PC2 gene.

Table 1. Concentrations of iNT (N- and C-terminal), iNN, CTiNN and iK6L in the brain of WT versus KO mice †

	iNT				
	C-terminal	N-terminal	iNN	CTiNN	iK6L
WT	48.0 ± 3.7	49.4 ± 2.4	31.9 ± 2.6	47.8 ±3.6	2.8 ± 0.3
PC2-/-	37.1 ± 3.0*	45.1 ± 2.5	14.6 ± 1.1*	43.3 ± 2.0	1.6 ± 0.4*
PC1+/-	46.6 ± 3.4	45.1 ± 2.1	32.4 ± 3.2	42.5 ± 1.2	2.7 ± 0.3
PC2-/-; PC1+/-	36.7 ± 1.9*	48.8 ± 2.4	14.3 ± 1.3*	46.8 ± 3.0	N.A.
PC7 -/-	54.8 ± 5.5	51.7 ± 2.8	32.6 ± 3.7	45.6 ± 1.2	2.9 ± 0.2

† Values are expressed in pMol/g and are the means ± 95% confidence intervals of 3-9 animals.
* $p<0.01$ when compared to corresponding WT values as determined using ANOVA followed by a Bonferroni's multiple comparison test. N.A., Not assayed.

FIGURE LEGENDS

Fig. 1. Diagrammatic representation of rat pro-NT/NN and of its major processing products in brain. The propeptide may be cleaved at any of the four Lys-Arg pairs of basic residues indicated. The sites recognized by the antisera K6L and E6I are also indicated (modified from Villeneuve et al., 2000b). SP, signal peptide.

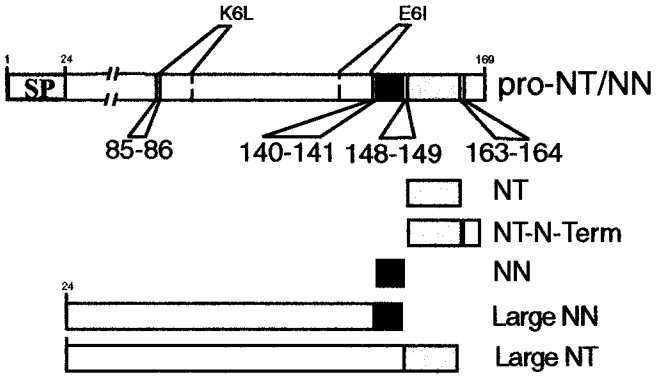

K6L
E6I
1
24
169
SP
pro-NT/NN
85-86
140-141
148-149
163-164
NT
NT-N-Term
NN
24
Large NN
Large NT

Fig. 2. Reverse-phase HPLC of acid extracts from brain of WT (A), PC1+/- (B), PC7-/- (C), PC2-/- (D) and PC2-/-; PC1+/- (E) mice. The amounts of iNT, iNN and CTiNN are indicated (pMol/fraction). CTiNN was obtained after subjecting all collected fractions to arginine-directed trypsin digestion and measuring their iNN content. Two major peaks eluting at 34-35 and 36-37 min and corresponding to NT and NN, respectively, were detected in all animals. A major immunopositive NN signal was also detected in fractions eluting at 53-56 min following trypsinization. Since no iNT was detected in these fractions, it can be assumed to correspond entirely to large NN. A small iNT peak was detected at 32-33 min. This immunoreactive peak was only detected with the N-terminal-directed antibody, suggesting that it corresponds to a C-terminally truncated or extended form of NT (N-terminal NT).

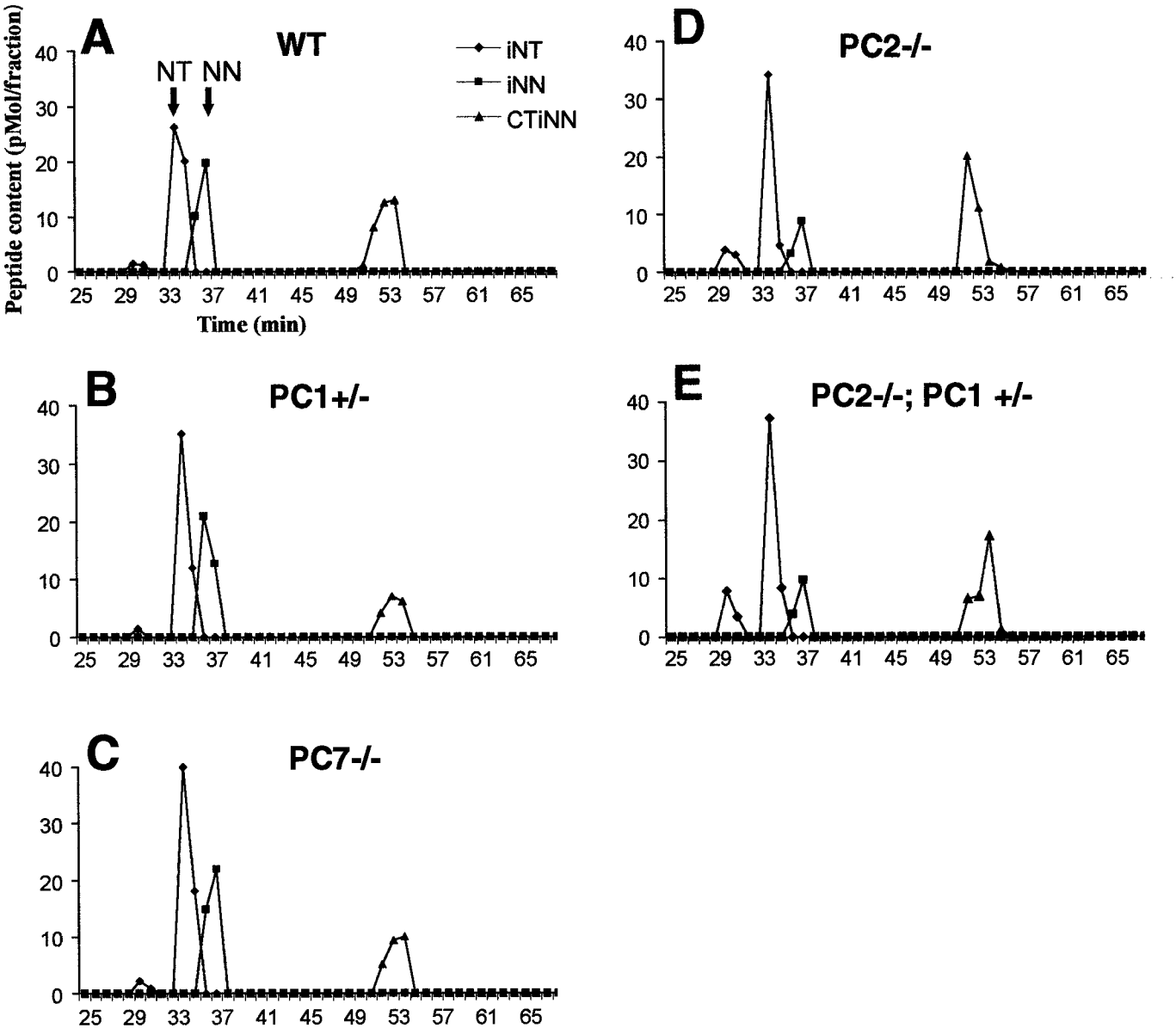

A
WT
NT NN
iNT
iNN
CTiNN
Peptide content (pMol/fraction)
Time (min)
B
PC1+/-
C
PC7-/-
D
PC2-/-
E
PC2-/-; PC1 +/-

Fig. 3. Relative amounts of pro-NT/NN products detected in the various animal models. The contents are expressed as a percent of CTiNN ± 95 % confidence intervals of 3-9 animals. Concentrations of maturation products are very similar in WT, PC1+/-, and PC7-/- animals but altered in PC2 KO as compared to WT mice. No large NT was detected in any of the samples. * $p<0.01$ when compared to corresponding WT values as determined using ANOVA followed by a Bonferroni's multiple comparison test.

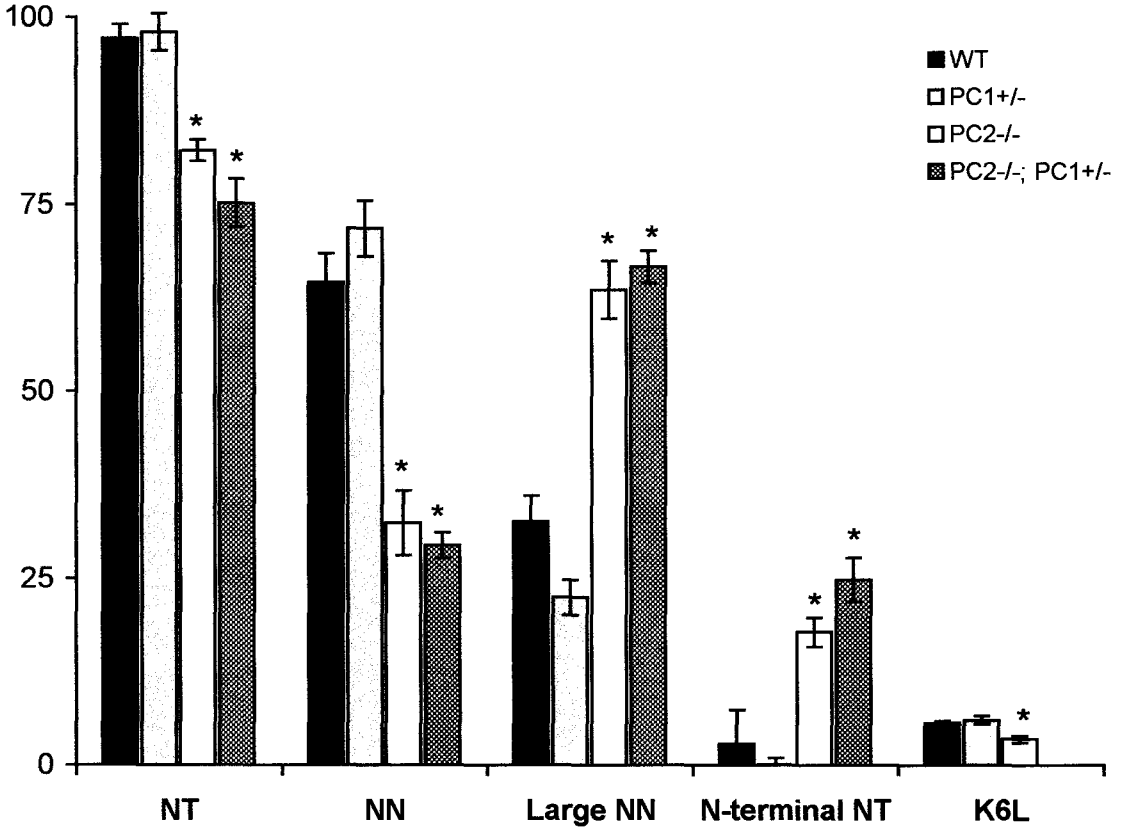

100
75
50
25
0
WT
PC1+/-
PC2-/-
PC2-/-; PC1+/-
NT
NN
Large NN
N-terminal NT
K6L

Fig. 4. Percentage of cleavage at the various pro-NT/NN pairs of basic residues in WT and PC2-/-, PC1+/-, PC2-/-; PC1+/-, and PC7-/- mice (± 95 % confidence intervals of 3-9 animals). Percentages were calculated by dividing the values of the mature products by the total content of the NT/NN precursor, as determined by CTiNN. * $p<0.01$ when compared to corresponding WT values as determined using ANOVA followed by a Bonferroni's multiple comparison test.

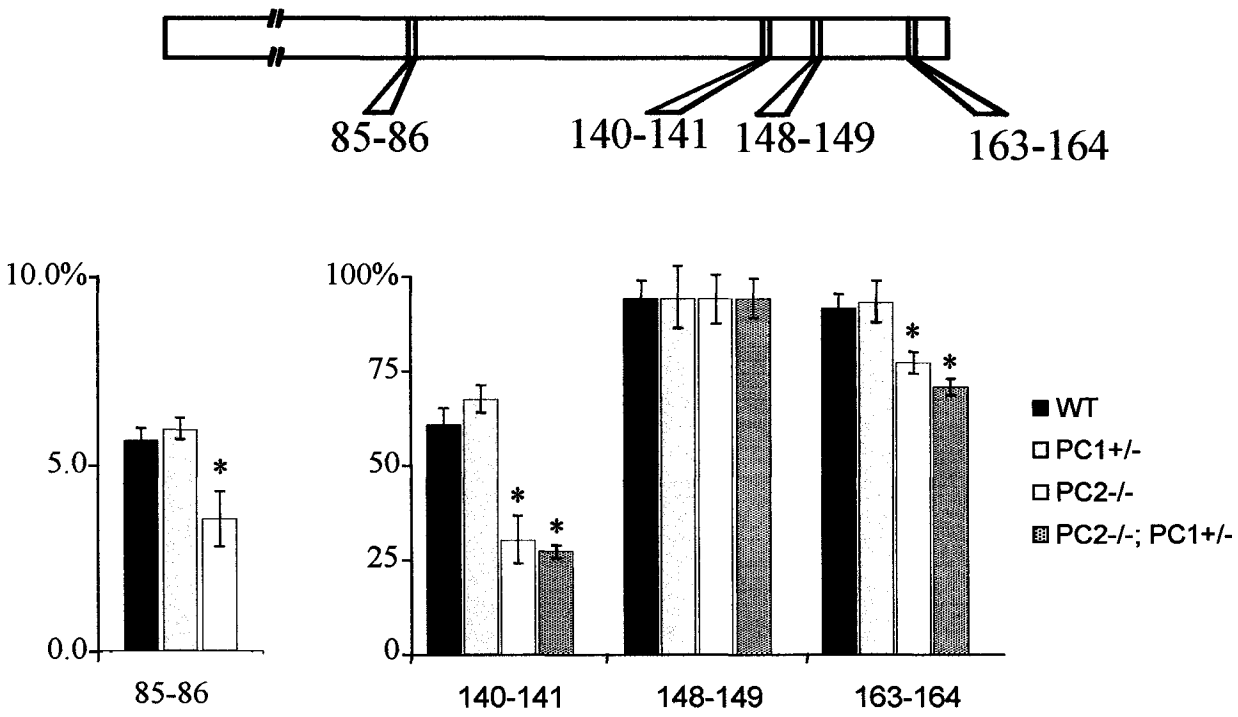

85-86
140-141
148-149
163-164
10.0%
5.0
0.0
85-86
100%
75
50
25
0
140-141
148-149
163-164
WT
PC1+/-
PC2-/-
PC2-/-; PC1+/-

Fig. 5. NT (A, A'), E6I (B, B'), and K6L (C, C') immunolabelling in the central nucleus of the amygdala (CeA) in WT versus PC2 null mice. For all three antigens, the immunoreactive signal is associated with a dense network of varicose axons (arrows). No detectable difference is noted in the overall immunostaining pattern and/or density between WT and PC2 null mice. Scale bar =40 μm.

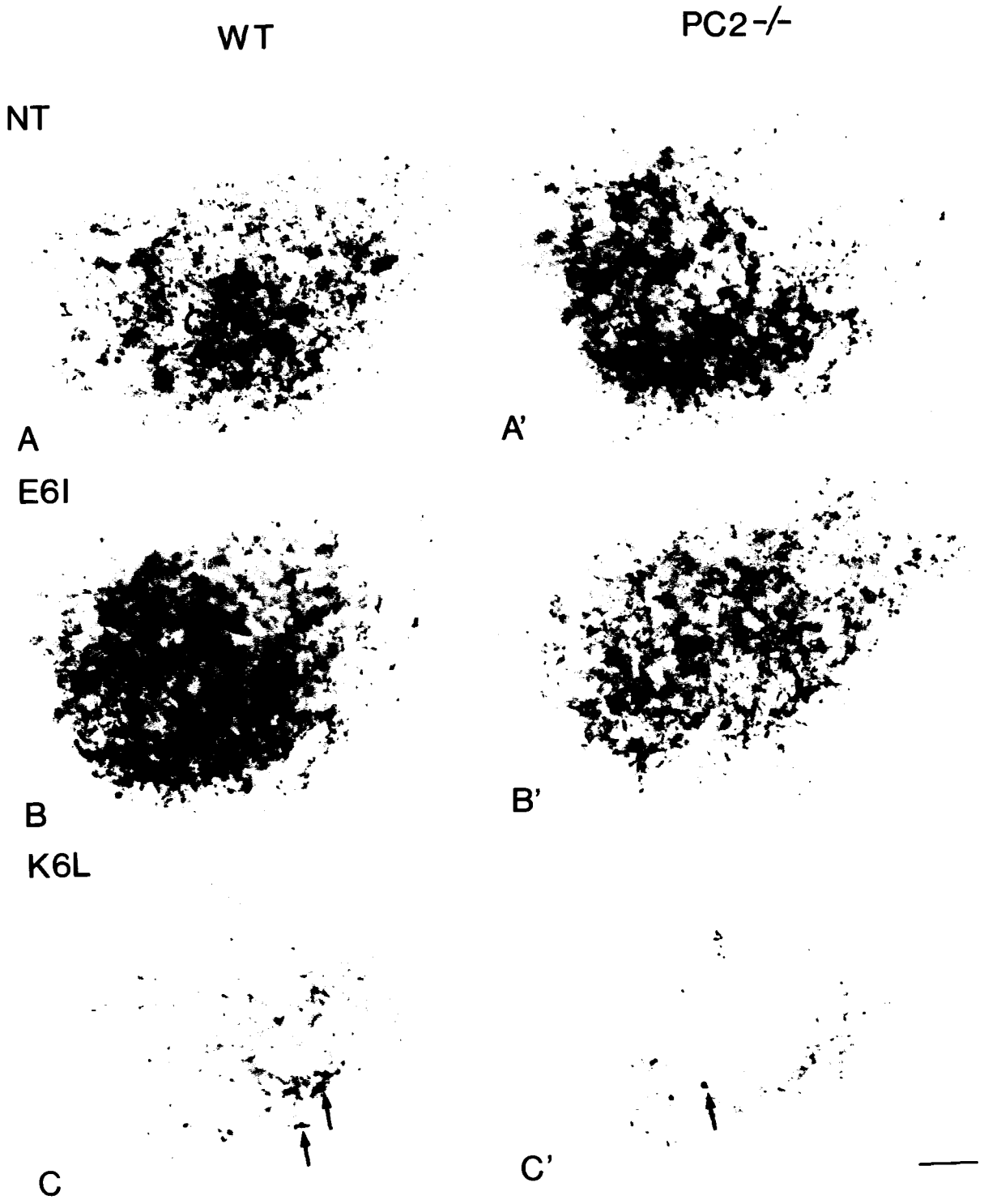

WT
PC2-/-
NT
A
A'
E6I
B
B'
K6L
C
C'

Fig. 6. Distribution of NT (A, A'), E6I (B, B'), and K6L (C, C') immunoreactivity in the medial preoptic area (MPA) of WT and PC2 null mice. Note that the overall density of the immunostaining is markedly reduced in PC2 null mice (A', B' and C') compared to WT (A, B and C). Examples of varicose axons are indicated (arrows). 3V, third ventricle Scale bar = 80 μm.

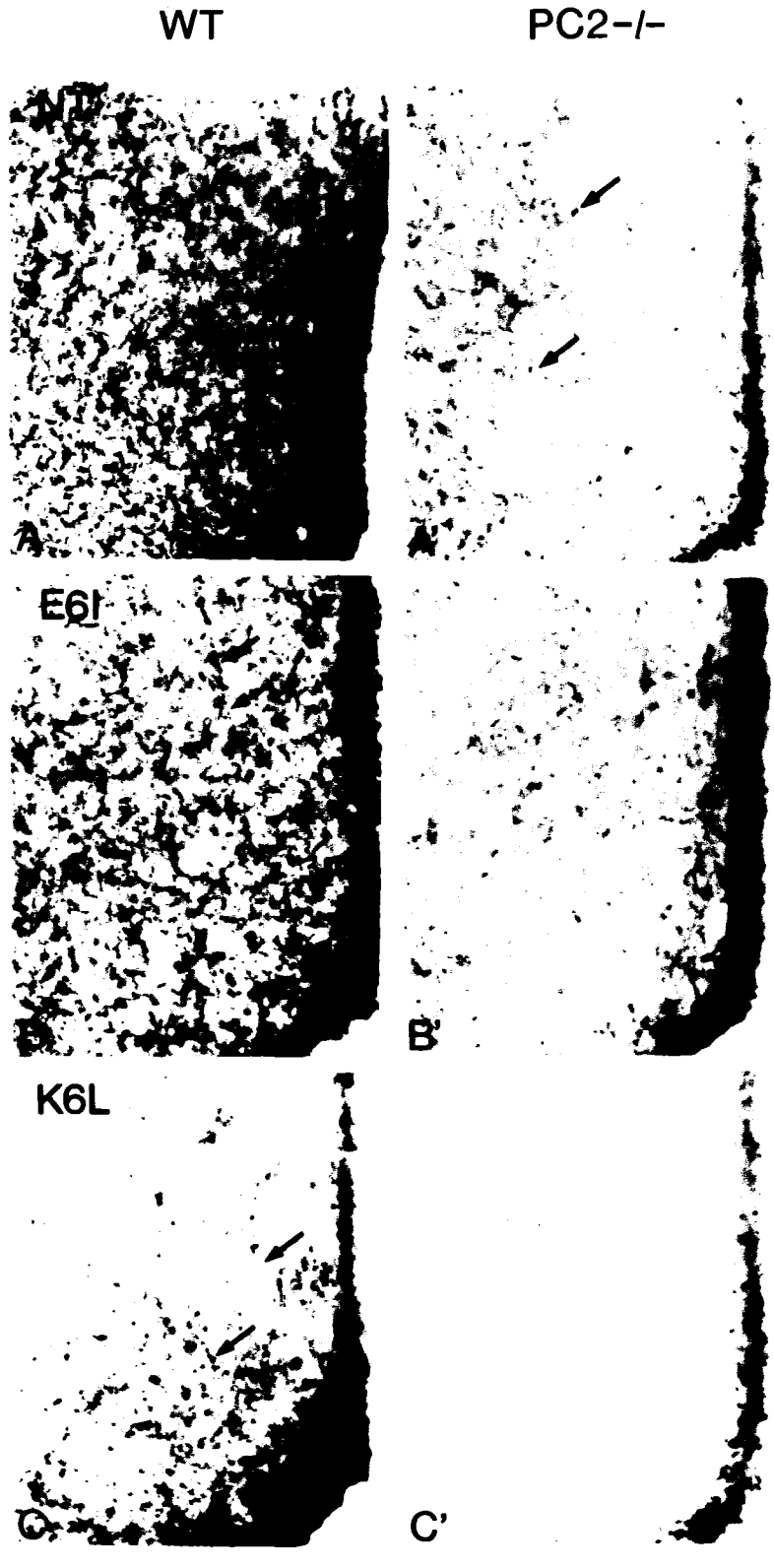

WT
PC2-/-
E6I
K6L
A
B'
C'

Fig. 7. Immunohistochemical staining for NT (A, A') and E6I (B, B') in the lateral hypothalamus (LH) of WT and PC2 null mice. Note the drastic decrease in both NT and E6I immunostaining in PC2 null mice compared to WT (A', B'). fo, fornix; mfb, medial forebrain bundle; ic, internal capsule; ot, optic tract; Scale bar = 102 μm.

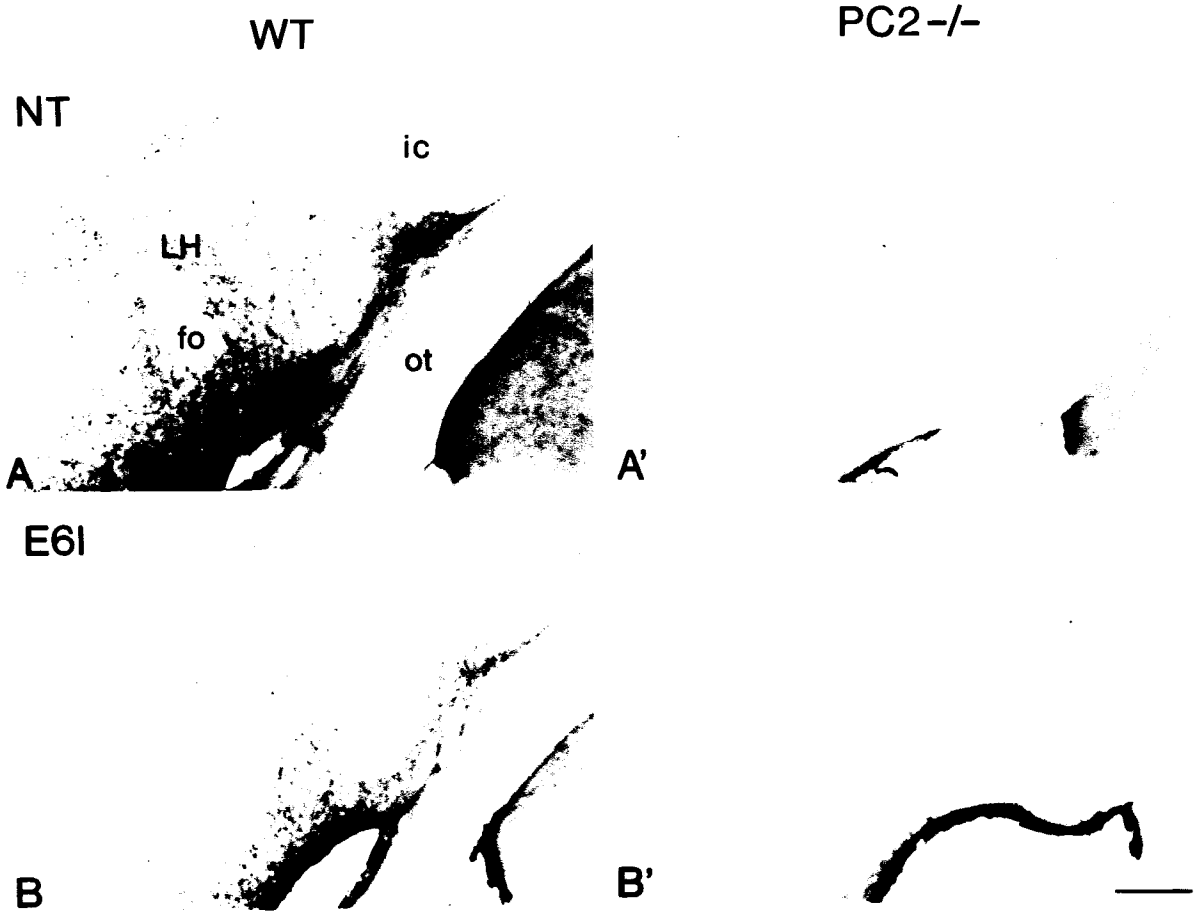
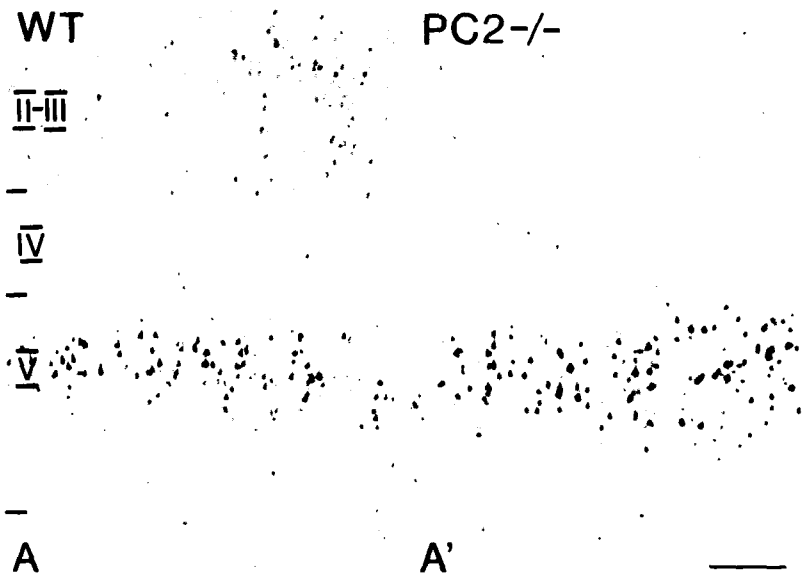

WT
PC2-/-
NT
ic
LH
fo
ot
A
A'
E6I
B
B'

Fig. 8. PC1 immunoreactivity in the magnocellular preoptic area (MCPOA) of WT (A) and PC2 null (B) mice. In both cases, PC1 immunoreactivity is mainly concentrated in nerve cell bodies but is also seen in neuronal processes. No difference in either the intensity of immunolabelling nor in the number of immunoreactive neurons is detectable between WT and PC2 null mice (arrows depict ventral border of basal forebrain). Scale bar = 83 μm.


MCPOA
B

Fig. 9. Distribution of PC5A-immunolabelled neurons in the cerebral cortex of WT (A) and PC2 null mice (B). In both WT and PC2 null mice, PC5A immunoreactivity predominated within the cell bodies and apical dendrites of pyramidal cells in layer V, although immunolabelled neurons are also evident in layer II-IV and VI. No detectable difference in either the density or distribution of the immunohistochemical signal is apparent between WT and PC2 null mice. Scale bar = 90 μm.


WT
PC2-/-
II-III
IV
V
A
A'

DISCUSSION

The present study was undertaken to determine the role of prohormone convertases PC1, PC2, and PC7 in the endoproteolytic maturation of pro-NT/NN in rodent brain. To this aim, we examined by radioimmunoassay and immunohistochemistry the patterns of pro-NT/NN processing in the brain of mice in which PC2 and PC7 genes had been totally inactivated or in which the PC1 gene had been partially knocked down.

Detailed biochemical analysis of pro-NT/NN maturation products in the brain of mutant vs WT mice revealed that total PC2 inactivation severely impaired pro-NT/NN conversion. Thus, we observed a 50 % decrease in the levels of the biologically active peptide NN and a concomitant increase in large NN in PC2-/- as compared to WT mice, suggesting that PC2 is critical for the *in vivo* production of this peptide in the brain. The absence of PC2 activity also led to a significant decrease in the production of mature NT and to the appearance of an immature, presumptive C-terminally-extended form of NT (N-terminal NT). This form of NT was virtually undetectable in the brain of WT animals and is unlikely to be endowed with biological activity since structure-activity studies have demonstrated that the C-terminal extension of NT results in a virtual loss of its biological activity (Kitabgi et al., 1985). PC2 inactivation also resulted in a 40 % decrease in the production of the K6L-immunoreactive peptide. The identity of this maturation product is unknown and its potential biological role remains to be investigated (de Nadai et al., 1994).

The significant changes observed here in pro-NT/NN maturation in PC2 null as compared to WT mice, together with earlier data showing that PC2 can process pro-NT/NN in recombinant cell systems (Rovere et al., 1996) and is extensively co-localized

with NT in rat CNS (Villeneuve et al., 2000b), provide strong evidence for the implication of PC2 in the *in vivo* processing of pro-NT/NN in rodent brain. To date, total inactivation of PC2 enzymatic activity has been shown to alter the processing of pro-insulin, pro-glucagon, pro-somatostatin (Furuta et al., 1997; Furuta et al., 1998), pro-dynorphin (Berman et al., 2000), pro-melanin concentrating hormone (Viale et al., 1999), pro-orphanin FQ/Nociceptin, pro-opiomelanocortin (Allen et al., 2001), and pro-SAAS (Sayah et al., 2001), all of which are routed through the regulated secretory pathway. By contrast, no difference was observed here in the maturation of pro-NT/NN between PC7 null and WT animals, suggesting that PC7 is not involved in the physiological processing of this precursor molecule. This result is in keeping with reports showing that PC7 is implicated in the processing of precursors routed towards the constitutive, rather than the regulated, secretory pathway (Seidah et al., 1996; Munzer et al., 1997).

In addition to PC2, the convertases PC1 and PC5A have both been shown to process the NT/NN precursor *in vitro* (Rovere et al., 1996; Barbero et al., 1998) and to co-localize with NT in various brain regions (Villeneuve et al., 2000b; Villeneuve et al., 2000a). These observations suggest that these two convertases are likely to also be involved in the processing of pro-NT/NN *in vivo* and might compensate to some extent for the lack of functional PC2 in the CNS of PC2-inactivated animals. Berman et al. (2000) showed that disruption of the PC2 gene does not lead to any change in the expression of PC1, PC5A, or furin in whole brain extracts. We further showed here by immunohistochemistry that neither the levels nor the distribution of PC1 and PC5A were altered in PC2 KO animals. It may therefore be surmised that the changes in pro-NT/NN processing observed in PC2 null mice are the resultant of the lack of functional PC2 and

of the remaining activity of PC1 and PC5A and possibly other PCs. The decreased production of NN and concomitant increase in large NN observed here in PC2 null as compared to WT mice is consistent with this interpretation. Indeed, studies in transfected PC12 cells have shown that whereas PC2 cleaves very efficiently and to the same extent the last three pairs of basic residues of pro-NT/NN, PC1 and PC5A cleave much less efficiently the Lys^{140}-Arg^{141} doublet that precedes and releases the NN sequence (Rovere et al., 1996; Barbero et al., 1998). PC1 and/or PC5 would therefore be less able to compensate for the loss of PC2 at this than at other cleavage sites in PC2 null animals. The greater efficiency of PC2 over PC1 at the EEVIKR^{141}-KIP sequence may be due to the fact that whereas PC2 favors a Glu at P6, PC1 does not (Apletalina et al., 1998).

We further observed that cleavage at the Lys^{163}-Arg^{164} residues in the C-terminus of the NT sequence was only moderately affected by PC2 inactivation and that cleavage at the Lys^{148}-Arg^{149} residues between NN and NT sequences was not affected at all, suggesting that the *in vivo* processing of these two sites is efficiently compensated by PC1 and/or PC5A in PC2 null mice. By contrast, the minor cleavage at the Lys^{85}-Arg^{86} residues observed in the brain of WT mice was decreased in PC2 null animals, suggesting that PC2 is active at this site *in vivo* and that its deletion is not efficiently compensated by other PCs. In fact, this doublet does not appear to be efficiently processed *in vitro* by any of the three PCs discussed here (Rovere et al., 1996; Barbero et al., 1998). Previous immunohistochemical studies have suggested that the K6L-generating cleavage is an event that occurs late in the regulated secretory pathway of pro-NT/NN expressing neurons the brain (Woulfe et al., 1994). This would fit with the implication of PC2 in this cleavage *in vivo*, since the enzyme has been shown to mediate late processing events

in the regulated secretory pathway (Lindberg et al., 1994; Malide et al., 1995; Villeneuve et al., 2000b).

Despite available evidence for the implication of PC1 in the processing of pro-NT/NN, the partial inactivation of PC1 (PC1+/-) had no effect on pro-NT/NN maturation profiles when performed alone and did not significantly modify the aberrant processing patterns observed in PC2 null mice when performed in combination with the inactivation of PC2. However, this lack of observable effect does not necessarily imply that PC1 does not play a role in the physiological processing of pro-NT/NN as the remaining allele may provide for sufficient PC1 activity to perform normal functions. Indeed, in partially knocked down PC2 mice, pro-dynorphin processing remains intact despite a 50 % decrease in enzyme activity, whereas it is severely impaired in the full knocked down mouse (Berman et al., 2000). Furthermore, the trend towards an exacerbation of the deficits observed in PC2-/-; PC1+/- animals as compared to PC2 null mice alone is suggestive of an interaction between PC1 and PC2. Anatomical evidence indeed suggests that PC1 and PC2 are co-localized in a subpopulation of central NT neurons (Winsky-Sommerer et al., 2000).

To characterize the deficits resulting from PC2 inactivation at the regional level, we compared the immunohistochemical distribution of pro-NT/NN maturation products between WT and PC2 null mice. The topographic distributions of NT-, E6I-, and K6L-immunoreactive axonal arbors observed here in WT animals are consistent with the reported distribution of these three antigens in rodent brain (Woulfe et al., 1994). Surprisingly, in most brain regions, there was little apparent decrease in the density of NT-, E6I-, or K6L-immunoreactive fibre networks between PC2 null and WT animals.

This was particularly surprising in the case of E6I immunoreactivity, which one would have expected to be the most affected given the loss of efficiency in the cleavage of the Lys^{140}-Arg^{141} doublet observed in whole brain extracts from PC2 KO mice. Therefore, immunohistochemistry may not afford sufficient sensitivity for the appreciation of moderate decreases in the levels of pro-NT/NN maturation products.

By contrast, immunoreactivity towards all three antigens was markedly decreased in selective brain regions including the medial preoptic area, lateral hypothalamus, and paraventricular nucleus of the hypothalamus. Furthermore, NT and E6I but not K6L exhibited lower immunoreactivity levels in the arcuate nucleus of PC2 null as compared to WT animals. These results differ from what might have been predicted on the basis of our findings in whole brain homogenates, in that the decreases in E6I immunoreactivity, and to a lesser extent in K6L immunoreactivity, should have been more pronounced than those of NT. Furthermore, they suggest that the massive depletion of E6I epitope observed in these regions cannot account by itself for the depletion in NN observed in whole brain, since, had this been the case, comparable decreases in NT levels should have been detected in whole brain extracts, which they were not. The most likely interpretation for our findings is therefore that in these regions, there are no other PCs co-localized with pro-NT/NN to compensate for the loss of PC2 activity. Admittedly, double localization studies showed fairly high proportions of NT nerve cell bodies co-localizing either PC1 or PC5A in all of these areas except in the paraventricular nucleus of the hypothalamus (Villeneuve et al., 2000b). However, since NT, E6I, and K6L immunoreactivity detected in these regions was mostly associated with axons, the proportions of NT nerve cell bodies co-expressing the convertases may not offer a

reliable index of the prevailing situation. Indeed, many of these axons originate from regions such as the nucleus accumbens and ventral tegmental area (Paklovits and Zaborszky, 1979), in which only a small proportion of NT neurons co-localize either PC1 or PC5A (Villeneuve et al., 2000). In any event, the present results suggest that the region-specific differences in pro-NT/NN processing observed in normal animals (Kitabgi et al., 1991; de Nadai et al., 1994) may be due to variable concentrations of the complement of other PCs, namely PC1 and PC5A, co-localized with PC2.

In conclusion, the results presented here provide strong evidence for a physiological role of PC2 in pro-NT/NN processing. They further demonstrate that this role is highly specific since it differently affects the various cleavage sites of pro-NT/NN, especially the one responsible for the release of mature NN. Finally, PC2-inactivation led to region-specific decreases in pro-NT/NN-immunoreactive products, suggesting that the importance of PC2 is region-specific but also that other convertases, namely PC1 and PC5A, are also involved in pro-NT/NN processing in the brain.

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Chapter Six

General discussion and conclusion

6.1 GENERAL DISCUSSION

In order to understand the physiological role of prohormone convertases in the brain, it is essential to know their distribution. The results presented in Chapter 2 provide the first description of the immunohistochemical distribution of PC5A in mammalian brain. We found that PC5A was exclusively associated with neurons, indicating that, as for PC1 and PC2, it is likely to process peptides routed to the regulated pathway in the brain.

At the regional level, PC5A was distributed throughout the rat brain with the highest levels found in the olfactory bulb, cerebral cortex, globus pallidus, septum and hypothalamic and thalamic nuclei. The convertases PC1 and PC2 have also been shown by *in situ* hybridization (Cullinan et al., 1991; Schafer et al., 1993) and immunohistochemistry (Winsky-Sommerer et al., 2000) to be selectively expressed by neurons distributed throughout the rat brain. The location of the convertases can point to potential peptide precursor target. For instance, the distribution of cholecystokinin in the neocortex and the thalamus (Innis et al., 1979) closely resembles that of PC2 and PC5A. Also, PC1, PC2 and PC5A have all been reported to be expressed in pro-somatostatin-rich areas such as the cortex, hippocampus, hypothalamus, and central nucleus of the amygdala. Numerous other examples could be given of the co-expression of one or several convertases with putative peptide substrates, as it appears that, overall, the distribution of these three enzymes overlaps with that of numerous peptide precursors. These results indicate that these three PCs are likely to play a role in the processing of numerous peptide precursors throughout the rat brain. In the case of PC5A, only two propeptides have so far been studied, namely pro-NT/NN (Barbero et al., 1998) and pro-

cholecystokinin (Cain et al., 2001) but our data indicate that the number of potential substrates is much higher in the brain.

To our surprise, the bulk of the immunoreactive signals for PC1, PC2 and PC5A was concentrated at the level of the perikarya. No PC5A immunolabelling was detected in nerve terminals whereas PC1- and PC2-immunopositive synaptic boutons were detected (Winsky-Sommerer et al., 2000), despite the fact that they constitute only a small fraction of total convertase immunoreactivity. The concentration of convertases PC1, PC2 and PC5A at the level of neuronal cell bodies is in sharp contrast to that of neuropeptides, which are concentrated in nerve terminals. This finding suggests that in the brain, the convertases PC1, PC2 and PC5A are not systematically secreted together with neurosecretory products but, rather, are either recycled to early compartments of the secretory pathway or simply retained there.

At the level of the perikarya, PC1 and PC5A immunoreactivities were concentrated in the Golgi apparatus. In both cases, the antibodies used detected predominantly mature, and not merely unprocessed forms of these enzymes in the brain (De Bie et al., 1996; Dong et al., 1997; Villeneuve et al., 2000; Winsky-Sommerer et al., 2000). It thus appears that both proteases are well positioned to start processing peptide precursors as early as in the Golgi apparatus. Early processing of peptide precursors, prior to packaging into secretory granules, has already been demonstrated *ex vivo* for POMC (Zhou et al., 1993; Zhou and Lindberg, 1993), pro-enkephalin (Johanning et al., 1996), pro-somatostatin (Lepage-Lezin et al., 1991), pro-thyrotropin releasing hormone (Nillni et al., 1993) and Aplysia's pro-egg-laying hormone (Sossin et al., 1990a). Recent evidence suggests that this may also be the case for pro-NT/NN since a mutated form of

the precursor, misrouted to the constitutive pathway of secretion, was nonetheless partially processed in β-TC7 cells (Feliciangeli et al., 2001). Early processing could theoretically provide for a segregation of PC1- and PC5A-generated products within different secretory granules, as previously demonstrated for the egg-laying hormone precursor in Aplysia (Sossin et al., 1990b). In the case of pro-NT/NN, however, this does not appear to be the case since the two major maturation products, NT and NN, were co-localized to the same secretory vesicles and are therefore probably co-released. It is possible, however, that other PC1- and PC5A-processed substrates undergo such segregation in the brain. Thus, the generally accepted concept that peptide maturation is initiated in the TGN but takes place predominantly in secretory granules may have to be revisited, as it does not appear to apply to all peptide precursors.

By contrast, PC2 co-localized poorly with the Golgi marker MG-160 and, instead, was concentrated in more distal compartments of the secretory pathway, in keeping with earlier reports of PC2 localization within mature secretory granules in different neuroendocrine cell lines (Lindberg et al., 1994; Malide et al., 1995; Muller et al., 1998) and with the fact that it gains enzymatic activity only at the level of the TGN and secretory granules (Benjannet et al., 1993; Shen et al., 1993; Zhou and Mains, 1994). PC2 is therefore well positioned to play a role in endoproteolytic maturation further along the secretory pathway than PC1 or PC5A.

The anatomical studies presented in Chapters 3 and 4 provide compelling evidence for a role of the convertases PC1, PC2 and PC5A in the processing of the NT/NN precursor in rat brain. Indeed, NT co-localized with all three PCs throughout the brain, and all three PCs had been shown to process pro-NT/NN in transfected cells (Rovere et

al., 1996; Barbero et al., 1998). However, the proportion of NT neurons colocalizing either PC1, PC2 or PC5A varied substantially between brain regions, which probably accounts for the regional variations in NT/NN ratios reported in the rat brain (Kitabgi et al., 1991; de Nadai et al., 1994). Differential processing of neuropeptides provides a critical mechanism through which cells may locally regulate the levels of specific peptides. Such a process had previously been reported for pro-somatostatin (Sawchenko et al., 1988), pro-dynorphin, POMC (Benjannet et al., 1991), pro-thyrotrophin-releasing hormone (Bulant et al., 1988), and pro-enkephalin (Zamir et al., 1984; Day and Akil, 1989). Interestingly, mature peptides arising from a common precursor molecule may exert different or even opposite effects. For instance processing of pro-orphanin FQ/Nociceptin can generate either nociceptin, which has anxiolytic properties, or nocistatin, which is anxiogenic (Jeanck et al., 2001). In the case of pro-NT/NN the effects of a differential processing of the precursor may be more subtle, as NT, NN, large NT and large NN all interact with the same receptors, albeit with different affinities and different resistance to metabolic degradation.

The variable intensity of PC1, PC2 and PC5A immunostaining observed between brain regions and even between zones within certain nuclei raises the possibility that the intracellular concentrations of the PCs relative to their putative substrates could also contribute to the regional differences observed in pro-NT/NN maturation profiles. However, in transfected cells overexpressing PC2, increasing the levels of expression of PC2 did not affect the maturation profile of pro-NT/NN (Rovere et al., 1996). Whether the same lack of effect of PC2 levels exist *in vivo* remains to be determined.

The extensive overlap observed here between NT and either PC1, PC2 or PC5A with NT suggests that in several brain areas, NT may co-localize with more than one PC within single neurons. Such redundancy would explain why the invalidation of PC2 resulted in only partial deficits in the processing of the NT/NN precursor, as it did in the case of many other propeptides investigated so far (Johanning et al., 1998; Viale et al., 1999; Berman et al., 2000; Allen et al., 2001; Sayah et al., 2001; Yasothornsrikul et al., 2001). Physiologically, the co-expression of multiple convertases presumably translates into synergistic processing of common substrates. There is experimental evidence for such a concerted action of convertases. For instance, POMC is first cleaved into β-LPH by PC1 and β-LPH is then cleaved into β-endorphin by the action of PC2 (Benjannet et al., 1991; Thomas et al., 1991; Zhou et al., 1993). A similar order of cleavage was reported for pro-insulin (Smeekens et al., 1992b; Rhodes et al., 1993). In this case, PC1 would act first to cleave the precursor at the Arg^{31}-Arg^{32} site, followed by PC2 acting at the Lys^{64}-Arg^{65} bond, thereby generating insulin and C-peptide. It has been speculated that the changes in conformation that accompany each cleavage may expose new sites to processing enzymes and thereby confer to "cocktails" of PCs properties that each PC may not have on its own (Smeekens et al., 1992a). For instance, a concerted action of PC2 with either PC1 or PC5A could lead to the processing of the Lys^{85}-Arg^{86} site, a doublet which was so far found to be resistant to any single PC alone (Rovere et al., 1996; Barbero et al., 1998) but which is cleaved in the brain of WT (de Nadai et al., 1994; Woulfe et al., 1994) but less so in PC2 KO animals.

In the case of the NT/NN precursor, studies in transfected cells have shown that PC1 and PC5A preferentially process dibasic residues Lys^{148}-Arg^{149} and Lys^{163}-Arg^{164},

whereas PC2 cleaves with equal efficiency all three last dibasics. It is therefore tempting to speculate, on the basis of our subcellular localization studies, that in NT-expressing neurons that contain PC1 or PC5A in addition to PC2, PC1 and/or PC5A would process the most C-terminal dibasic residues at the level of the Golgi, whereas PC2 would process in part or totally the remaining N-terminal dibasic residues in post-Golgi compartments. Given the differential subcellular localization of these PCs, this selectivity could translate into a sequential processing of the NT/NN precursor. Comparison of the relative immunolabelling densities of pro-NT/NN maturation products at the level of cell bodies and nerve terminals had already suggested the existence of such a sequential cleavage of the different dibasic sites of pro-NT/NN in the course of axonal transport from the cell body to the nerve terminals (Woulfe et al., 1994). It was then proposed that the processing of the NT/NN precursor was mediated by sequential cleavage 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), then by Lys^{140}-arg^{141} bond (which liberates NN and effectively exposes the E6I sequence) and finally the Lys^{85}-Arg^{86} site (which exposes K6L). The results obtained in PC2 KO mice support this hypothesis since the most affected dibasic residues were Lys^{85}-Arg^{86} and Lys^{140}-Arg^{141}. The fact that the cleavage of Lys^{163}-Arg^{164} was also affected indicates that PC2 is also implicated in the processing of this site, presumably whenever it has not been processed in the early secretory pathway by PC1 or PC5A. Given the high degree of overlap observed between the convertases throughout, a concerted action of several convertases on a common precursor molecule within single cells may be common in the processing of precursors, which, like pro-NT/NN, contain multiple cleavage sites.

Neuropeptides exhibit a high degree of plasticity in terms of their levels of expression, depending on the physiological/pathological state of the organism or even the pharmacological agents to which it is exposed. Given the putative roles of the convertases in the processing of peptide precursors, it is conceivable that their expression might be regulated in parallel with that of their substrates *in vivo*. Indeed, upon colchicine treatment, we observed a selective increase in the immunostaining intensity of PC5A, but not of PC1 or PC2, in NT-rich areas. Although colchicine treatment is not a physiological stimulus, the coincidental up-regulation of both PC5A and NT at the level of neuronal perikarya indicates that a physiological coupling between NT and PC5A may exist. We investigated this idea further by monitoring the expression of PC5A, but also that of PC1 and PC2, in animals that had been treated with the D2 antagonist haloperidol, a drug which had been shown to induce a selective increase in the number of NT-immunoreactive neurons in the neostriatum and nucleus accumbens (Eggerman and Zahm, 1988). However, no concomitant changes in the immunoreactive signal for any of the convertases tested was observed in these experiments, even when the animals were treated with high doses chronically. Likewise, we did not observe any changes in the topological distribution or in the immunolabelling intensity of PC1 or PC5A in the brain of PC2 null mice, indicating that the expression of these two PCs is probably not linked to that of PC2. By contrast, PC1 and PC2 mRNA are strikingly up-regulated in response to haloperidol and down-regulated upon exposure to bromocriptine (Day et al., 1992), in parallel with mRNA for POMC and corticotrophin-releasing hormone in the pituitary (Day et al., 1992). The changes observed in the levels of expression of PC1 and PC2 were region-specific since, in the intermediate lobe, haloperidol increased both PC1 and

PC2 whereas in the anterior pituitary, only PC2 was up-regulated. In the same study, the effects of thyroid hormones on PC1 and PC2 mRNA levels were investigated. It was found that PC1 and PC2 mRNA levels were increased 5- to 9-fold in the pituitary of hypothyroid animals. In contrast to its effects on PC1 and PC2 expression, hypothyroidism decreased NT/NN mRNA levels in rat pituitary. It therefore appears that the expression of at least some of the convertases may be coupled to that of their substrates in response to specific pharmacological or physiological challenges.

Glucocorticoids have been documented to be potent modulators of the expression of mRNA encoding for pro-NT/NN and of the convertases PC1 and PC2. Thus, dexamethasone exposure leads to an up-regulation of PC1 and/or PC2 mRNA in AtT-20, PC12 and rMTC 6-23 cells (Day et al., 1992; Rovere et al., 1996) and that of pro-NT/NN mRNA in PC12 cells. In addition, rats treated chronically with corticosterone have their levels of NT mRNA increased in both the periventricular and rostral arcuate nuclei of the hypothalamus, but not in any other brain region (Nicot et al., 1995). The expression of pro-NT/NN and that of PC1 and/or PC2 may therefore be coupled, *in vivo*, in at least a subpopulation of neurons. This could in turn modify the pro-NT/NN processing profile in the affected regions.

Analysis of pro-NT/NN maturation profile in PC2 null mice has allowed us to better define the role of this protease in the processing of pro-NT/NN in the CNS. We observed marked decreases in the levels of NN and iK6L and a moderate decrease in NT levels in PC2 null mice compared to WT, due to selective impairments in the cleavage of the sites N-terminal to NN and K6L, and to a lesser extent, of the site C-terminal to NN. These results provide compelling evidence for a physiological role of PC2 in the processing of

the NT/NN precursor in the rodent brain. However, the fact that pro-NT/NN processing was only partly impaired following PC2 invalidation supports the hypothesis that other convertases, namely PC1 and PC5A, are implicated in the processing of pro-NT/NN. We observed by immunohistochemistry that the abolishment of PC2 activity did not result in a uniform alteration in pro-NT/NN processing throughout the brain since marked decreases in NT-, E6I- and K6L-immunoreactivity were noted in the medial preoptic area, paraventricular and arcuate nuclei of the hypothalamus and lateral hypothalamus but not in other areas. These results may reflect the regional variations in the degree of co-expression of PC1 and/or PC5A with PC2 in NT neurons.

In addition to the degree of co-expression of NT with members of the convertase family, other factors are likely to be important in the processing of the NT/NN precursor. Examples of determinant factors include local differences in the intracellular concentrations of Ca^{++} (Shennan et al., 1995) and pH (Shennan et al., 1995; Lamango et al., 1999) or region-specific degree of co-expression of the PCs with helper/inhibitor proteins such as 7B2 (Lindberg et al., 1995; Zhu et al., 1996) and pro-SAAS (Fricker et al., 2000; Sayah et al., 2001). Indeed, although both pro-SAAS and 7B2 are present in all PC1- and PC2-containing neurons in the brain, their levels of expression are highly variable (Seidel et al., 1998; Feng et al., 2001; Lanoue and Day, 2001; Sayah et al., 2001). The different ratios of 7B2/PC2 and pro-SAAS/PC1 are likely to influence the proportions of enzymatically active convertases.

As discussed in Chapter 5, total invalidation of PC2 enzymatic activity had previously been shown to alter the processing of numerous peptides in the brain, including pro-dynorphin (Berman et al., 2000), pro-melanin concentrating hormone

(Viale et al., 1999), pro-orphanin FQ/Nociceptin, and pro-opiomelanocortin (Allen et al., 2001). Since the normal maturation of these peptide precursors leads to the release of biologically active peptides, the investigation of how specific behaviour are affected by the invalidation of PC2 should provide further insights of the convertases effects at the behavioural and/or physiological level. For instance, we detected marked decreases in the levels of immunoreactive NT/NN maturation products at the level of the hypothalamus, a key centre for the regulation of pituitary hormones secretion. This will likely give rise to numerous hormonal defects and probably accounts for the impaired fertility of PC2 null mice (Seidah et al., personal communication). An interesting behaviour to study would be sensitivity to pain since the processing of pro-dynorphin is affected in the PC2 knockout mice. These studies could provide us with a better understanding of the role of the PCs and of the maturation products arising from peptide precursors at the behavioural level, and may even help the future identification of diseases arising from deficient prohormone processing.

Overall, our results illustrate the complexity of the process of propeptide maturation by enzyme members of the convertase family in the mammalian brain. In order to further our understanding of the mechanisms involved in this process, it will be important to focus future studies on how this maturation operates *in vivo*. One possible approach will be to further characterize propeptide processing defects in available convertase KO mouse models. However, in such a paradigm, it is possible that the effects of invalidating any given PC are masked due to compensation by other PCs or by modulation of the effects of PC inhibitors. More importantly, they evade the developmental effects which possibly occur in classical KO. Although we have controlled for the expression of PC1

and PC5 in the PC2 knockout mouse brain and did not find any detectable difference, the processing of pro-SAAS is impaired in PC2 null mice (Sayah et al., 2001) and this may affect the enzymatic activity of PC1 in these animals. The development of KO mouse models inactivated for several PCs simultaneously might overcome at least some of the inherent drawbacks of gene targeting models. They could also provide better insight of the interactions between the convertases.

Alternative approaches include the use of antisense oligonucleotides or of specific protease inhibitors (whenever more specific ones will be available; Fugere et al., 2001), or the development of inducible KO for given PCs. These three approaches could allow the specific targeting of a convertase to a defined brain region or organ. They could be particularly useful for the elucidation of the role of PC1, PC5A and furin since mice inactivated for these three PCs are not viable (Roebroek et al.,1998; Seidah et al., personal communication). These approaches, in complement with anatomical of the type presented here, could provide a powerful means to determine the specific roles of convertases in the processing of a precursor and the consequences of their invalidation on specific behaviors and physiological responses.

6.2 SUMMARY AND CONCLUSION

The results of the present study can be summarized as follows:

1) PC5A protein is extensively distributed in the rat brain where it is selectively associated with neurons, suggesting that it plays a role in the endoproteolytic maturation of numerous precursor proteins.

2) The extensive immunohistochemical co-localization of the prohormone convertases PC1, PC2 and PC5A with pro-NT/NN provides strong evidence for the implication of these convertases in the maturation of this precursor *in vivo* and suggests that at least in some regions, these proteases may act jointly.

3) The subcellular localization of the presumptive enzymatically active forms of PC1 and PC5A in the Golgi apparatus provides evidence for an early implication of these enzymes in the regulated secretory pathway of neurons. Conversely, PC2, which is concentrated in post-Golgi compartments, would act later along the regulated secretory pathway.

4) The altered processing of the NT/NN precursor observed in PC2 null mice suggests that PC2 plays a crucial role in the physiological processing of pro-NT/NN in the brain. Our results suggest that this role is quite specific since the cleavage sites of the NT/NN precursor are affected differentially in the mutant animals; the cleavage site responsible for the release of NN was the most affected one, whereas cleavage of the site N-terminal to NT was not affected at all. Finally, impairments in pro-NT/NN processing were considerably more pronounced in certain brain regions than others, implying that compensation by other convertases varies among brain regions.

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